OSMO-REGULATION OF *DUNALIELLA TERTIOLECTA* AND REGULATORY MECHANISM FOR GLYCEROL SYNTHESIS UNDER OSMOTIC STRESS

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NATIONAL UNIVERSITY OF SINGAPORE

2014

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Zhao Ran

10 Aug 2014

ACKNOWLEDGEMENTS

It has been my dream to be a scientist since I was in primary school. Finally I am getting to it nearer and nearer after several years walking and running, until now. At this exciting point and moment of my PhD journey, I would like to express my sincere appreciation to all my mentors and friends who have been accompanying and enlightening me all the way.

Especially, I would like to express my heartfelt gratitude to all my lab members, and staffs and students in Department of Microbiology, all of whom had a hand in guiding me, encourage me and bring me to this memorable milestone of my life.

Firstly, I would like to appreciate my supervisor A/P Lee Yuan Kun. He not only helped me a lot guiding my research journey, but also gave me a lot of useful suggestions for life. He is always so nice when talking to us, and ready to discuss with us. Prof Lee, thank you very much to provide me the opportunity to work in the algae group, and thank you very much for your kind supervision in my PhD studies. It would not have been possible for me to reach this point without all your guidance, advice, encouragement and support.

Also, I would like to thank the lab officer Mr Low Chin Seng, who has offered me a lot of tips on conducting experiments, and for the colorful life in Singapore. As it is said, Mr Low, you are really capable and

important for the laboratory, and “like an important gear in a piece of machinery, you keep the laboratory well-oiled and running as it should”.

Thirdly, I would like to thank the post-doctoral research fellows, Dr Fang Lei, Dr Yvonne Chow, Dr Shen Hui and Dr Ng Yi Kai. Thank you very much for sharing me your expertise, your precious experience and suggestions. To my laboratory mates, who are also post-graduate students, Ms Ng Hui Ping Daphne, Ms Lin Hui Xin, Ms Yao Li Na, Ms Radiah Safie, Mr Kelvin Koh, Mr Kenneth Tan and all the other guys and girls in the water group, thank you very much for the cheerful times we shared. I will always remember the days we did experiments together especially with my research partner Ms Ng Hui Ping Daphne, hung out eating and shopping together and celebrated the monthly birthdays together. I feel so fortunate staying in this big family and spending the over four years together with all of you.

Lastly, I am deeply indebted to my family, especially my parents for their patient love and sustained encouragement which enabled me to complete this work. I would like to thank my dearest friends as well, especially those we came to NUS as post-graduate students together, and I really appreciate the accompanied and delighted times with you.

To those who I have inadvertently missed out here, I would like you to know that you are important too.

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SUMMARY

This thesis consists of mainly two parts of work: description of the osmo-regulation in the marine microalga *Dunaliella tertiolecta* under different osmotic stress conditions; identification and characterization of the two key genes involved in the osmo-regulatory mechanisms for glycerol production by *D. tertiolecta* under osmotic stress.

The eukaryotic and photosynthetic microalgae *Dunaliella* respond to osmotic stress by varying the glycerol content. Physiology study on the cellular behavior revealed that *D. tertiolecta* rapidly adjust its cell size, in addition to the variation on intracellular glycerol concentrations in response to changes in external osmotic stress. Moreover, glycerol production by *D. tertiolecta* was found to be proportional to the external salinity (osmolarity), and higher glycerol productivity was observed with higher salinity. It was concluded that the intracellular glycerol accumulated in *D. tertiolecta* functioned as the osmolyte maintaining osmotic balance in the osmo-regulation process, while the continuously produced abundant extracellular glycerol can be a stable and effective additional carbon sink for the photosynthetic CO_2 assimilation.

Due to the limited genetic information of *Dunaliella*, the two cDNA sequences *DtPFK* and *DtMAPK*, which encode key enzymes involved in the regulation of glycerol synthesis, were identified in *D. tertiolecta* using the Rapid Amplification of cDNA Ends (RACE) technology in order to verify the osmo-regulatory mechanisms for glycerol production in *D. tertiolecta*. However, gene knock-down of *DtPFK* by RNA

interference indicated that the glycolysis and pentose phosphate pathway had minor contribution to the glycerol synthesis in *D. tertiolecta*, and *DtPFK* may not be involved in the regulation of glycerol synthesis as it had been suggested previously. On the other hand, characterization of *DtMAPK* suggested that the expression of *DtGPDH*, which encodes the enzyme responsible for glycerol synthesis, was regulated by *DtMAPK*. This is the first time to show a direct relation between *DtMAPK* expression and glycerol production in *D. tertiolecta*, demonstrating a MAPK involved regulatory mechanism for *D. tertiolecta* glycerol synthesis. These studies shed light on the molecular basis for the osmo-regulation in *D. tertiolecta*.

This project provided evidence that *D. tertiolecta* can be used for the sustainable capture of atmospheric carbon dioxide. Based on molecular study results, future work aspects have been prospected to fulfil the information that *DtMAPK* mediated regulating pathway, and to achieve transgenic *D. tertiolecta* strains with enhanced CO_2 fixation ability and glycerol productivity via gene modification approaches.

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Gene and protein names

AMT	Adenine methyltransferase gene promoter
ble	Bleomycin (zeocin) resistance gene
bar	Phosphinothricin acetyltransferase (bialaphos resistance gene)
CAT	Chloramphenicol acetyltransferase
CDPK	Ca^{2+}-dependent protein kinase
DsMPK	*D. salina* mitogen-activated-protein kinase
DtGPDH	*D. tertiolecta* glycerol-3-phosphate dehydrogenase
DtMAPK	*D. tertiolecta* mitogen-activated-protein kinase
DtPFK	*D. tertiolecta* phosphofructokinase
DtTUB	*D. tertiolecta β-tubulin* gene
GFP	Green fluorescent protein
EGFP	Enhanced green fluorescent protein
GPDH	Glycerol-3-phosphate dehydrogenase
GUS	β-glucuronidase encoding gene
MAPK	Mitogen-activated-protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
PFK	Phosphofructkinase

Chemicals and reagents

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl sulfoxide

DHAP	Dihydroxyacetone phosphate
EDTA	Ethylenediaminetetraacetic acid
Glycerol-3-P	Glycerol-3-phosphate
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KCl	Potassium chloride
LB	Luria Bertani medium
LiAc	Lithium acetate
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Disodium hydrogen phosphate
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfate
Tris	Tris (hydroxymethyl) aminomethane
TAE buffer	Tris acetate electrophoresis buffer
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
β-ME	β-mercaptoethanol (2-mercaptoethanol)

Units and measurements

atm	Standard atmosphere(s)
Da	Dalton
kDa	Kilodalton

g	Gram(s)
mg	Milligram(s)
µg	Microgram(s)
ng	Nanogram(s)
h	Hour(s)
min	Minute(s)
s	Second(s)
M	Molar per litre
mM	Milimolar per litre
µM	Micromolar per litre
nM	Nanomolar per litre
mol	Molar(s)
mmol	Milimolar(s)
µmol	Micromolar(s)
nmol	Nanomolar(s)
µm	Micrometer(s)
L	Litre(s)
ml	Millilitre(s)
µl	Microlitre(s)
nm	Nanomolar(s)
rpm	Revolution per minute
U	Unit(s)
v/v	Volume per volume
w/v	Weight per volume
°C	Degree Celsius
K	Kelvin

Others

bp	Base pair(s)
kb	Kilo base pair(s)
CDS	Coding sequence
chl	Chlorophyll
DNA	Deoxyribonucleic acid
NUP	Nested Universal Primer A
OD_{600}	Absorbance at wavelength 600 nm
PCR	Polymerase chain reaction
QRT-PCR	Quantitative real-time PCR
PPP	Pentose phosphate pathway
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
RNAi	RNA interference
mRNA	Messenger ribonucleic acid
UTR	Untranslated regions
UPM	Universal Primer A Mix
p	Significance level
R^2	Coefficient of determination

LIST OF PRIMERS

Primer name	Sequence (5' to 3')	Annotation
For RACE		
DtPFK-dgF1	5'-GCCCAAGTCGATAGACAACGAC-3'	Degenerate primers for cloning of *PFK* fragments
DtPFK-dgF 2	5'-GCCTGGTGAAGCTCATGGG-3'	
DtPFK-dgR1	5'-TGGCCCAGCACCTTGCAGTA-3'	
DtPFK-dgR 2	5'-TTCTGCCCTGCACCCTCCG-3'	
DtPFK-gsF1	5'-CTCCATGGCATCAGGTGTTGTG-3'	*DtPFK* 3'-RACE
DtPFK-gsF2	5'-ATGTGTGCCTCATCCCTGAGATT-3'	
DtPFK-gsR1	5'-CAATATCTGCCAGGATGGGGTT-3'	*DtPFK* 5'-RACE
DtPFK-gsR2	5'-CCCTTTGAAGCACCCTTCACAA-3'	
DtMAPK-dgF1	5'-AGGAGCATACGGTGTGGTTTG-3'	Degenerate primers for cloning of *MAPK* fragments
DtMAPK-dgF2	5'-AAAGTGGCCATCAAGAAAAT-3'	
DtMAPK-dgR1	5'-TAGCTCAGGGGCTCTGTACCA-3'	
DtMAPK-dgR2	5'-TAGTCCTTGCCGGGGAACA-3'	
DtMAPK-gsF1	5'-TGCCAAACGCACACTGCGTG-3'	*DtMAPK* 3'-RACE
DtMAPK-gsF2	5'-CGCCTGATGTGGTGCGAGGC-3'	
DtMAPK-gsR1	5'-ATCGCGGTGCAGGATGGCAG-3'	*DtMAPK* 5'-RACE
DtMAPK-gsR2	5'-CCACACCGTATGCTCCTTTGCCA-3'	
UPM-Long	5'-ctaatacgactcactatagggcAAGCAGTGGTATCAACGCAGAGT-3'	Universal Primer A Mix
UPM-Short	5'-ctaatacgactcactatagggc-3'	
NUP	5'-AAGCAGTGGTATCAACGCAGAGT-3'	Nested Universal Primer A
For expression analysis (QRT-PCR)		

DtPFK-rtF	5'-CTCCATGGCATCAGGTGTTGTG-3'	Primers for *DtPFK* expression
DtPFK-rtR	5'-CAATATCTGCCAGGATGGGGTT-3'	
DtTUB-rtF	5'-CAGATGTGGGATGCCAAGAACAT-3'	Primers targeted against *DtTUB* sequence
DtTUB-rtR	5'-GTTCAGCATCTGCTCATCCACCT-3'	
DtGPDH-rtF	5'-ATTAACCTGCTTGCGGATGT-3'	Primers for *DtGPDH* expression
DtGPDH-rtR	5'-CATAGCTGCTGGCAATCAAA-3'	
DtMAPK-rtF	5'-AGCCCCTGAGCTACTGCTCTCAT-3'	Primers for *DtMAPK* expression
DtMAPK-rtR	5'-GTTCAGCTGGTGCACGTAGTCCT-3'	

For vector construction

AMT-F-KpnI	5'-aaaGGTACCATCAGTAATGTGTTAATTGC-3'	Cloning primers for *AMT* promoter
AMT-R-XhoI	5'-ccgCTCGAGTTAAATAATATATAGTGTATTTTAG-3'	
ble-F-XhoI	5′-AAACTCGAGATGGCCAAGCTGACCAGC-3′	Cloning primers for *ble* gene
ble-R-ClaI	5′-CACATCGATTTAGTCCTGCTCCTCGGC-3′	
DtPFK-SF-PstI	5'-AACTGCAGGCTTTGGCTTTGAGACAGCA-3'	Sense fragment of *DtPFK* for RNAi construct
DtPFK-SR-BamHI	5'-CGGGATCCGGATGGCACGGATCATGTAA-3'	
DtPFK-AsF-ClaI	5'-CCATCGATGGATGGCACGGATCATGTAA-3'	Anti-sense fragment of *DtPFK* for RNAi construct
DtPFK-AsR-EcoRI	5'-GGAATTCGCTTTGGCTTTGAGACAGCA-3'	
DtMAPK-SF-PstI	5'-AACTGCAGAGTCCATCAACTATGAGGCCG-3'	Sense fragment of *DtMAPK* for RNAi construct

DtMAPK-SR-BamHI	5'-CGGGATCCTCGTACACCACATACAGGTCC-3'	
DtMAPK-AsF-ClaI	5'-CCATCGATTCGTACACCACATACAGGTCC-3'	Anti-sense fragment of *DtMAPK* for RNAi construct
DtMAPK-AsR-EcoRI	5'-CGGAATTCAGTCCATCAACTATGAGGCCG-3'	

For genotyping PCR

Ble-gen-F	5'-GGAGCGGTCGAGTTCTGG-3'	Primers for *D. tertiolecta* mutants
Ble-gen-R	5'-CTCGCCGATCTCGGTCAT-3'	

For colony PCR and sequencing

AMT-seq-F	5'-GGTTGTTGCGAGAAATTTTG-3'	Forward primer for constructs containing *AMT*
Ble-seq-F	5'-AACTGCGTGCACTTCGTGG-3'	Forward primer for constructs containing *ble*
GUS102-R	5'-AACGGTTTGTGGTTAATCAGG-3'	Reverse primers at the 5'-end of GUS fragment in pGreen-0229 HY104 (RNAi construct backbone)
PGP2	5'-CCTTATCGGGAAACTACTCACAC-3'	Reverse primer for pGreen-0229 HY104 vector (RNAi construct backbone)
T7	5'-TAATACGACTCACTATAGGG-3'	Forward primer for pGEM®-T Easy vector (T/A cloning)
SP6	5'-ATTTAGGTGACACTATAGAA-3'	Reverse primer for pGEM®-T Easy vector (T/A cloning)

CHAPTER 1 INTRODUCTION AND OBJECTIVES

1.1 Introduction

1.1.1 Energy shortage and global warming

In human history, consumption of fossil fuels has been on the rise. Supply of fossil fuels is limited and burning of fossil fuels introduces severe environmental problems, especially global warming. According to reports from United Nation's Intergovernmental Panel on Climate Change (IPCC), the average planet's surface temperature has a clear and unmistakable upward trend since the late 1800s. This climate change has major impact on water resources, energy supply, transportation, agriculture, and ecosystems. Emission of carbon dioxide (CO_2) and other global warming gases, which are also known as greenhouse gases, is the major cause of global warming. Thus reducing CO_2 emission and recycling the atmospheric CO_2 are two effective processes combating climate change.

1.1.2 Microalgae can be used as CO_2 remover and source of renewable energy

Study on photosynthetic microalgae has become a hot spot recently, not only because of the potential to use microalgae as bio-factories to produce alternative clean and renewable energy, namely biodiesels (i.e. fatty acid methyl ester), but also because these microalgae are phototrophic organisms which capture atmospheric CO_2 to enhance the

cycling of carbon in nature. Usage of photosynthetic microalgae has many advantages compared to plants. First of all, microalgae have much faster growth rate and higher photosynthetic efficiency than higher plant (Chisti 2007), which means they can utilize and transform more solar energy into chemical energy in biodiesels in a much faster way. Secondly, they are farm-land friendly and can be grown on non-arable land. Since available farm land is already limited, using microalgae can save more space for crop growing. For some marine species, they can even grow in sea water. In addition, microalgae can adapt to various extreme environments. The diversity of microalgae makes it possible to produce various valuable bio-products including biofuel precursors and nutraceuticals (Kay 1991; Lamers, Janssen et al. 2008; Gouveia and Oliveira 2009).

1.1.3 Advantages of using the algae *Dunaliella*

Dunaliella cells are unicellular, photosynthetic and motile biflagellate, which widely exist in marine waters. *Dunaliella* species belong to the phylum Chlorophyta, class Chlorophyceaee, order Chlamydomonadales and family Dunaliellaceae. Some widely known species such as *D. salina*, *D. tertiolecta*, *D. primolecta*, *D. viridis* and *D. parva* have been assessed recently (Borowitzka and Siva 2007). Those species of *Dunaliella* have been well studied for their unique features such as halo-tolerance and ability to accumulate significant amounts of valuable bio-products such as β - carotene and glycerol (Ben-Amotz and Avron 1981; Kleinegris, Janssen et al. 2011).

Being the only eukaryotic, photosynthetic and unicellular organisms tolerant of various salt concentrations (Ben-Amotz and Avron 1981), *Dunaliella* are also important in basic research as models for salt adaptation mechanism study. These single cell organisms can thrive in environment with a wide range of salt concentrations from 0.05 M to saturated salinity (Ben-Amotz and Avron 1981; Ben-Amotz and Avron 1990). Unlike most photosynthetic plant or microalgal cells, *Dunaliella* lacks a rigid cell wall. *Dunaliella* cell is only enclosed by a single layer plasma membrane (Ben-Amotz and Avron 1983), which confers its flexibility in adapting to the changing environmental osmotic condition. Furthermore, *Dunaliella* require high salt concentration for optimal cell growth and metabolic activities. They adopt unique osmo-regulation mechanisms to cope with the changing extracellular osmotic pressure. Osmo-responses of several *Dunaliella* species have been well studied on cell morphology and structure, as well as cell metabolic activities (Chen and Jiang 2009).

Among them, *D. tertiolecta* has a cell size of 10−12 μm, containing a single cup-shaped chloroplast and a central pyrenoid surrounded by starch granules. *D. tertiolecta* is fast growing and has a high CO_2 sequestration rate (Jiang, Yoshida et al. 2012). It may be a potential candidate for photosynthetic CO_2 assimilation.

1.1.4 Glycerol as the osmolyte and an additional carbon sink

Dunaliella adapt to various osmotic stresses by varying glycerol content. Glycerol could account for 50% of cell dry weight in some

species of *Dunaliella* under certain conditions (Ben-Amotz and Avron 1990). The accumulated massive glycerol confers *Dunaliella* another commercial potential to be used as a source for glycerol production (Ben-Amotz and Avron 1981; Chisti 2007). Glycerol is a simple polyol compound with wide applications in food industry, pharmaceutical and personal care and other aspects. Currently glycerol is mainly produced as the by-product of biodiesel production or as a co-product of soap-making. However, the crude product is of variable quality, and the purification process is expensive. As a result, production of cleaner glycerol is required with lower cost.

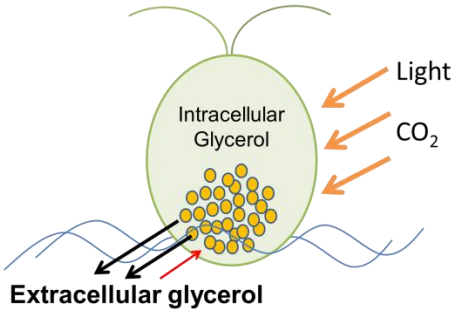

Figure 1-1 Glycerol as the osmolyte and additional carbon sink
While the intracellular glycerol content reaches an osmotic balance with the external osmolarity, extra glycerol is produced to media in the form of extracellular glycerol.

Dunaliella fix carbon and use glycerol as the carbon pool, thus it could be used as a candidate for CO_2 removal in the atmosphere. Intracellular glycerol is accumulated as the osmolyte to cope with external osmotic pressure, while extracellular glycerol is an additional carbon sink for carbon fixed by photosynthesis (Figure 1-1). The amount of extracellular glycerol could reach 4 times as much as the combined intracellular carbon fraction (biomass, intracellular glycerol, storage starch and other intracellular materials), since the extracellular

glycerol is not limited by cell bio-volume (Chow, Goh et al. 2013). Besides, glycerol is more stable than biomass and is used as substrate for many industrial products. Therefore, extracellular glycerol can serve as a significant carbon sink for fixed CO_2. *Dunaliella* can be an efficient candidate for photosynthetic CO_2 capture and potential source for glycerol production.

1.2 Research Objectives

1.2.1 Gaps and significances

Dunaliella species are ideal for assimilation of atmospheric CO_2 due to their fast growth speed and high photosynthetic efficiency, and can produce commercial glycerol as an effective carbon pool. In order to enhance the carbon flux from fixed CO_2 to glycerol, more information on *Dunaliella* osmo-regulation mechanisms is required and that how those pathways can regulate glycerol production needs to be understood. The accumulation of glycerol in *D. salina* in response to osmotic stresses is well studied (Chen and Jiang 2009). However, regulation of glycerol production in the fast growing *D. tertiolecta* is less known. The observation that cell size of *D. tertiolecta* does not vary as much as that of *D. salina* in response to change of medium salinity (Lin, Fang et al. 2013) suggests that *D. tertiolecta* may have different osmo-regulatory pathways. Moreover, few studies were conducted on the molecular basis of osmo-regulatory mechanisms in *D. tertiolecta*, and genetic information of *Dunaliella* is still limited so far. Therefore, identification and characterization of those core molecules involved in

potential regulatory pathways would facilitate the understanding of *Dunaliella* osmo-regulation on its glycerol production. The gained knowledge will make it possible to enhance the carbon flux from CO_2 fixed by photosynthesis to glycerol in *D. tertiolecta* from a genetic engineering approach.

1.2.2 Objectives of the study

The major objective of this project is elucidation of osmo-regulation mechanisms in *D. tertiolecta*. Those studied aspects include following parts:

1) Physiological study on cellular behavior of *Dunaliella tertiolecta* LB-999 strain under various osmotic stress conditions, including gross structural changes and metabolic activities. *Dunaliella* structural changes are described by variation of cell size and cell shape. *Dunaliella* metabolic changes are illustrated by carbon allocation under osmotic stress, which includes photosynthetic activities, glycerol accumulation and starch storage. It is of great importance to decide whether these synthetic activities are involved in osmo-regulatory behavior of *D. tertiolecta*, especially the osmo-regulation on glycerol production.

2) Metabolic pathways related to glycerol synthesis. Glycerol is synthesized via the glycerol cycle in *Dunaliella*. Glycerol, starch and biomass are important carbon-rich products. Basal

level of biomass is necessary for cell growth and other metabolic activities. In addition to photosynthetic CO_2, starch mobilizations (including glycolysis and its bypath pentose-phosphate pathway) also provide carbon for glycerol synthesis (Goyal 2007; Goyal 2007), suggesting that glycolysis and its bypath may be related to the regulation of glycerol synthesis in *D. tertiolecta*.

3) Other possible pathways that might be involved in glycerol synthesis. In addition to controlling at metabolic levels, glycerol production can also be regulated by other mechanisms, such as a cell signaling pathway. A HOG-like MAPK cascade is presumed in this project as the osmo-regulatory signaling pathway of *Dunaliella*. The purpose is to demonstrate whether this signaling pathway is related to the regulation of glycerol synthesis in *D. tertiolecta*.

As a major strategy to look into these possible pathways involved in osmo-regulation in *D. tertiolecta*, rate-limiting enzymes, checkpoints and core activators in those pathways were studied. The phosphofructokinase (PFK) in glycolysis and pentose phosphate pathway, the mitogen-activated-protein-kinase (MAPK) in the presumed HOG-like signaling pathway, and the glycerol-3-phosphate dehydrogenase (GPDH) in glycerol synthesis cycle were studied in this project, as these molecules might modulate glycerol production *D. tertiolecta* in responses to osmotic stress.

Investigation on these key enzymes as the molecular basis for osmo-regulatory mechanisms of *D. tertiolecta* is of great values as *D. tertiolecta* is a good model for study of salt-tolerance and stress-survival mechanisms in plants and crops. Besides, gene-level studies on these molecules would allow generation of transgenic *Dunaliella* strains with enhanced carbon fixation ability and glycerol productivity.

CHAPTER 2 LITERATURE REVIEW

2.1 Microbial Halotolerance and Halophilism

2.1.1 Description of halotolerance and halophilism

Osmolarity is a crucial environmental factor in determining the physiology function of microbial cells. Normally, salt (NaCl) is the osmolyte that determines the osmolarity of water environment. Most of the microbes can only survive in the range of fresh water to marine salinity, but some of the microbes tolerate or even prefer to live in high salt concentration. The ability of organism to tolerate the hyper-saline environment is called halotolerance (Brown 1976).

Similar to halotolerance, the microbes with halophilism grow in high salt concentration. However, halophiles are extremophilic organisms that prefer or require the extreme environment of high salt, which means they are not able to grow in fresh water or low salt environments. These organisms are usually found in evaporation ponds or salt lakes. Halotolerant organisms do not require salt but can still grow under saline conditions.

2.1.2 Categorizations and applications of halophiles

Most of the halophiles are within the Archaea domain. Certain bacteria and eukaryotes that belong to algae and yeast are also halophiles. For example, the bacterium *Halobacterium salinarium*, and cyanobacteria *Aphanothece halpphytica* are both prokaryotic halophiles, and

eukaryotic examples include the algae *Dunaliella* (Borowitzka and Brown 1974) and fungus such as *Wallemia ichthyophaga* (Gostincar, Lenassi et al. 2011).

Halophiles are usually categorized according to their extent of tolerance to salt concentration. Slight halophiles prefer 0.3 to 0.8 M (1.8 - 4.7%. Seawater is about 0.6 M or 3.5%), moderate halophiles 0.8 to 3.4 M (4.7 to 20%), and extreme halophiles 3.4 to 5.1 M (20 to 30%) NaCl (Ollivier, Caumette et al. 1994).

Halophilic microorganisms are an important part of the ecosystem. Their functions as well as applications have been summarized by researchers (Tafreshi and Shariati 2009; Oren 2010). The halophilic algae *Dunaliella* can provide food for a population of wild birds and contribute to eco-balance in the saline lakes and bottom of the sea. Besides, halophilic *Dunaliella* have shown the ability to clean up the heavy metal pollution, especially for the treatment of waste water with high salt concentrations (Takimura, Fuse et al. 1996; Hirata, Tsujimoto et al. 2001). Products of halophiles have been widely used in food processing and fermentation, such as soy sauce making (DasSarma and DasSarma 2001).

2.2 Discovery and Habitats of the Microalgae *Dunaliella*

The biflagellate microalgal genus *Dunaliella* has been widely studied as model organisms for the study of osmo-regulation, carotenoid

production, and photosynthesis under extreme conditions (Oren 2005). The first description of *Dunaliella* was in 1838, which reported an unicellular biflagellate and red-colored organism found in concentrated brines (Dunal 1838), and the organism has been known today as *Dunaliella salina*. Later in 1905, in-depth description of this organism was made (Heidelberg 1905; Teodoresco 1905), and *Dunaliella* was firstly named in honor of its discoverer Dunal as a new genus (Teodoresco 1905).

Subsequently, Teodoresco distinguished two different species *D. salina* and *D. viridis* (Teodoresco 1906), where *D. salina* was described having larger cells and could produce massive carotenoid pigments, which resulted in brightly red-colored cells under suitable conditions, while *D. viridis* could not produce such red cells under such conditions. However, some researchers doubted that the green cells and red cells were belonging to two different species. They considered the color change might be caused by the environmental salt concentration (Labbé 1921; Labbé 1921), or the red and the green cells were just the different forms or stages of the same species (Blanchard 1891; Heidelberg 1905).

Additional species were reported during the last century following the recognition of *D. salina* and *D. viridis*, and characterized based on morphological and physiological features. It has been agreed that *Dunaliella* species are ubiquitous in saline environments. Many species of *Dunaliella* are extremely halotolerant, such as *D. salina* and *D. viridis*,

and present widely in evaporation saltern ponds and salt marshes around the world. In addition to those typically halophiles, several marine species which have never been found in hyper-saline environments were also grouped in the genus of *Dunaliella*. Besides, freshwater species have also been described in the genus of *Dunaliella* although they had been proposed to be a different genus at the beginning (Melkonian and Preisig 1984; Preisig 1992). Another interesting species that is not a halophile in this genus is *D. acidophila*. As suggested by its name, *D. acidophila* was isolated from acidic waters and grows optimally in extremely low pH conditions (Gimmler and Weis 1992).

From 1999, with the aid of sequencing 18S rRNA gene and internal transcribed spacer, species with similar morphological and physiological descriptions, such as *D. salina*, *D. parva* and *D. bardawil*, were differentiated (Olmos, Paniagua et al. 2000). It is noteworthy that the morphological-physiological attributes in the earlier studied were not always comparable to the results from molecular studies (Oren 2005). The molecular study makes it much easier to revise those species with poor classification or erroneous characterization and to identify new species.

Dunaliella species are considered to play a major role in the ecosystems as primary producers of photosynthetic biomass especially in hyper-saline environments (Ben-Amotz, Polle et al. 2009) and they often give rise to blooms in salt lakes such as the dead sea (Oren and

Shilo 1982; Oren, Gurevich et al. 1995; Oren 2005). Among the diverse species of *Dunaliella*, the marine chlorophyte *D. tertiolecta* are widely found in oceans and sea water. Cells of *D. tertiolecta* (Figure 2-1) have an ellipsoidal, oval or pyriform shape with two flagella at one end and contain a single cupshaped chloroplast and a central pyrenoid surrounded by starch granules. *D. tertiolecta* cells are generally 5-18 µm in length and 4.5-14.0 µm wide, which are smaller than the cell size of halophilic species such as *D. salina* and *D. bardawil*. However, *D. tertiolecta* has a faster growth rate, a lower optimal salinity and high CO_2 sequestration rate (Jiang, Yoshida et al. 2012), thus it has gained attentions in laboratory study such as since its first report in 1959 (Butcher 1959).

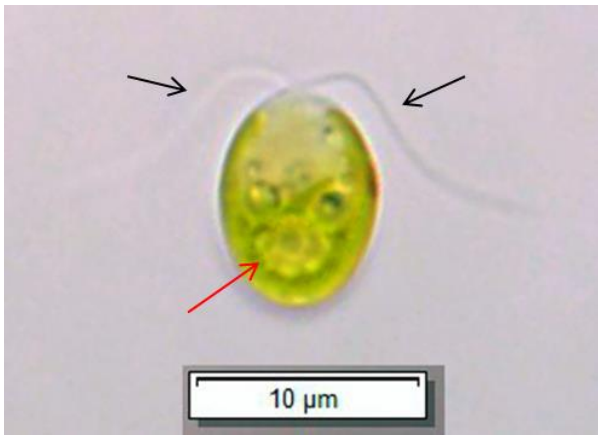

Figure 2-1 *D. tertiolecta* in 2 M NaCl ATCC-1174 DA medium
Logarithmic phase of *D. tertiolecta* cells growing in 2 M NaCl ATCC-1174 DA medium were photographed at 1000 times magnification under a light microscope (Olympus, Japan). Black arrows indicate the two flagella and red arrow indicates the central pyrenoid with the cup-shaped chloroplast.

2.3 Industrial and Commercial Bio-products of *Dunaliella*

Dunaliella has gained various values in different areas due to the diversity of this genus and the unique features of adaptation into various environments. Valuable bio-products have been studied in *Dunaliella* for their industrial and commercial potential.

2.3.1 Natural β - carotene

The commercial production of β - carotene is the most successful application of *Dunaliella*. The massive β - carotene accumulated by *D. salina* or *D. bardawil* under appropriate conditions has led to industrial production of natural β – carotene (Borowitzka and Borowitzka 1989; Del Campo, Garcia-Gonzalez et al. 2007; Raja, Hemaiswarya et al. 2007; Oren 2010).

The common nutrition supplement β - carotene is naturally found in carrot, spinach and broccoli etc. It has been used as a food coloring agent, as an additive to cosmetics, and as an anti-oxidative healthy food product. Various strains of *D. salina* have been grown commercially in mass cultures for the production of natural β - carotene (Borowitzka 1999), which occupies approximately 10% of commercial market apart from the chemically synthesized β - carotene (Raja, Hemaiswarya et al. 2007).

The mechanisms of β - carotene production by *D. salina* have been well known. The accumulated β - carotene protects cells against the injury caused by high intensity irradiation (Borowitzka and Borowitzka 1989). Ben-Amotz and Avron evaluated the factors that might affect β - carotene production, including the nutrients and salinity of the culture medium, as well as the light intensity (Ben-Amotz and Avron 1983). It was found that limitation of nutrients could promote the production of β - carotene. It should be noted that *D. salina* was also referred to as *D. bardawil* in some early reports (Ben-Amotz and Avron 1983), since the two species had quite similar morphological-physiological descriptions until that *D. bardawil* was differentiated from *D. salina* with the aid of molecular studies.

Generally, optimized β - carotene production has been achieved by treatment with high salt stress and nitrogen deficiency in areas of high solar radiation (Ben-Amotz and Avron 1983; García-González, Moreno et al. 2005). Large-scale cultivation of *D. salina* is based on autotrophic growth in media containing inorganic nutrients with CO_2 as exclusive carbon source. Therefore, *Dunaliella* production farms are normally built in areas close to salt water (sea water), usually with warm and sunny weather and abundant non-farmable land.

2.3.2 Microalgal biomass

Microalgal biomass can be used for human nutritional supplement or animal feed due to the high content of digestible protein and long-chain omega-3 fatty acids, which is substitute for fish meal and fish oils

(Benemann 2013). As a widely distributed microalga, *Dunaliella* has also gained interest as whole cells supplements beside the successful usage of *D. salina* for the commercial production of β - carotene.

D. salina has been cultivated in relatively small-scale currently and used as high value human nutritional products. As a “whole-food” supplement, the whole dried *D. salina* powder and capsules are commercially available which provides a range of trace minerals and other nutrients, such as β - carotene, with antioxidant, anti-inflammatory and photo-protection activity. It is also claimed that supplementation with *D. salina* may assist in prevention of a range of conditions including cardiovascular disease, some cancers and exercise-induced asthma, and the antioxidants in *D. salina* may provide some protection against exposure to the sun and other sources of radiation. Studies have suggested that humans who consumed a diet high in carotenoid-rich vegetables and fruits have a lower incidence of several types of cancer and cardiovascular disease . Most convincing reports have suggested such a direct connection. However, experimental nutrition and medical studies with natural carotenoids originating from the algae have been limited, and it lacks specific information on the possible medical contribution of whole dried *D. salina* which contains natural carotenoids, isomers of carotenoids, and carotenoid fatty acid esters.

2.3.3 Biofuel production

Table 2-1: Yield of bio-oils produced from a variety of organisms (Avagyan 2008)

Source	Gallons of oil (per acre per year)
Corn	15
Microalgae	
--Based on actual biomass yields	1850
--Theoretical laboratory yield	5,000-15,000
Oil palm	635
Rapeseed (canola)	127
Soybeans	48
Sunflower	102

It has been estimated (Avagyan 2008) that microalgae could produce 5000-15000 gallons of oil per acre per year. As shown in Table 2-1, the productivity of microalgae is 40-120 times more than canola and 8-25 times that of palm oil. Thus microalgae have been considered as one of the best sources for biodiesel and biofuel production using microalgal farming has been studied to satisfy the massive demand on biofuels using limited land resources without causing potential biomass deficit (Li, Horsman et al. 2008; Schenk, Thomas-Hall et al. 2008).

By comparing the culture and biofuel producing efficacy of different microalgae such as *Isochrysis galbana*, *Pavlova lutheri*, *D. salina* and *Nannochloropsis* sp., *D. salina* has been considered as promising feedstock for biodiesel production with a yield of 66% by direct

transesterification of microalgae biomass (Shenbaga Devi, Santhanam et al. 2012; Kumar, Balavigneswaran et al. 2013). This suggests that *Dunaliella* is a good candidate for production of clean and renewable energy.

2.3.4 Glycerol

Glycerol is a simple polyol compound with wide applications in food industry, pharmaceutical and personal care and other aspects. So far the glycerol in the market is mainly produced as the by-product of biodiesel production via transesterification (Figure 2-2) or as a co-product of soap-making. However, the crude product is of variable quality, and the purification process is expensive. As a result, cleaner production of glycerol is required with lower cost.

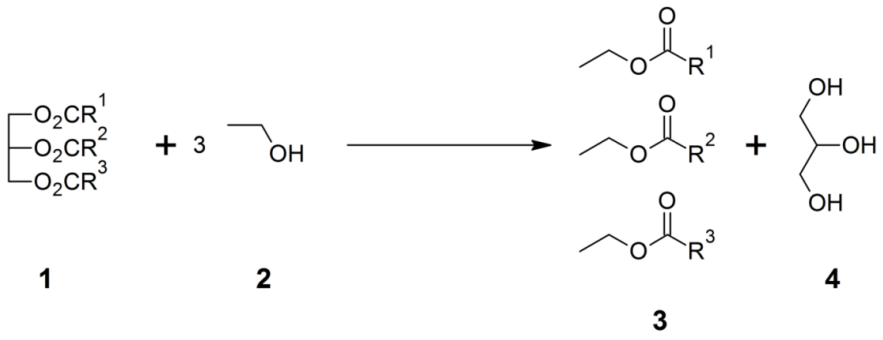

Figure 2-2 Glycerol prodcution via transesterification
Triglycerides (1) are treated with an alcohol such as ethanol (2) with catalytic base to give ethyl esters of fatty acids (3) and glycerol (4).

Dunaliella have no cell wall, while the halophilic species require high salt concentrations for optimal growth and other cellular activities (Borowitzka and Siva 2007). To tolerate high osmolarity, significant amounts of glycerol are accumulated as the compatible solute in the halophilic *Dunaliella* to provide protection against the environmental

osmotic pressure (Ben-Amotz and Avron 1973; Borowitzka and Brown 1974). Due to the great value of glycerol, *Dunaliella* glycerol production and the related photosynthesis have become interesting study directions. Glycerol is synthesized by a novel glycerol cycle (Figure 2-3) in *Dunaliella*, which is a two steps catalyzing pathway involving conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate and then to glycerol. To date, molecular investigations have been made at the protein level of the specific enzymes involved for the formation of glycerol (Sadka, Lers et al. 1989; Liska, Shevchenko et al. 2004). The enzyme glycerol 3-phosphate dehydrogenase (GPDH), which is also known as DHAP reductase, catalyzes the rate-limiting step and is indispensable for glycerol synthesis. Isoforms of GPDH encoding gene have been reported in *D. salina* and *D. tertiolecta* (Ghoshal, Mach et al. 2002; He, Qiao et al. 2007; Yang, Cao et al. 2007; He, Meng et al. 2009). The features of GPDH from *D. salina* suggest that the phosphoserine phosphatase domain may function as glycerol 3-phosphatase and the NAD+ -dependent GPDH may catalyse the step from DHAP to glycerol directly (He, Qiao et al. 2007; Yang, Cao et al. 2007; He, Meng et al. 2009). Unique isoforms of GPDH identified in *D. tertiolecta*, which are responsible for glycerol synthesis, were suggested having different properties than other chloroplast isoforms from plants and algae (Ghoshal, Mach et al. 2002; He, Qiao et al. 2007), providing a possible explanation for the fast glycerol synthesis in *Dunaliella*.

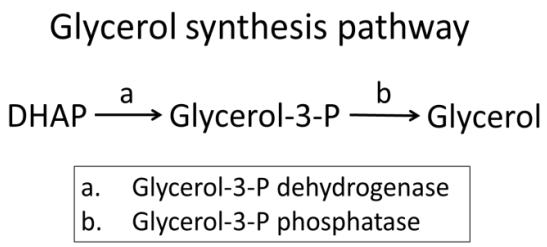

Figure 2-3 Glycerol synthesis pathway
DHAP: dihydroxyacetone phosphate; Glycerol-3-P: glycerol-3-phosphate.

Dunaliella adapt to various osmotic stresses by varying the glycerol content (Avron 1986). The unique fast glycerol synthesis pathway in *Dunaliella* offers an opportunity for biological production of glycerol in large amounts. Glycerol could account for as high as 50% of cell dry weight in some species of *Dunaliella* under certain conditions (Ben-Amotz and Avron 1990). The massive glycerol accumulation confers *Dunaliella* another commercial potential as a glycerol production source (Ben-Amotz and Avron 1981; Chisti 2007).

2.4 Biotechnological applications of *Dunaliella*

2.4.1 Waste water treatment and pollution control by *Dunaliella*

In addition to their valuable by-products, microalgae also play important roles in pollution control. *Dunaliella* has been widely used in wastewater treatment due to their wide range of habitats and tolerance to high salt environments.

Moreover, treatment of wastewater can be combined together with CO_2 mitigation as well as with production of microalgal biofuel and biomass fir animal feed, as the process is not as strict as biomass production for

human diet. This strategy can significantly lower the cost as it allows minimized use of freshwater. However, contamination is always a major concern associated with using wastewater for microalgae cultivation, thus appropriate pretreatment need to be done to remove sediment and to deactivate (sterilize) the wastewater (Noue and Pauw 1988; Munoz and Guieysse 2006; Li, Horsman et al. 2008).

2.4.2 *Dunaliella* as an eukaryotic host for recombinant protein production

Recombinant protein is a multi-million dollar market. It is widely applied in food industry, pharmaceutics production and nutrition supplements (Barzegari, Hejazi et al. 2010; Rasala and Mayfield 2014). In order to produce protein efficiently with high yield and low cost, it is necessary to choose a proper host species. Currently, several hosts have been used in the recombinant protein production such as *E. coli*, yeast and mammalian cell culture. *E. coli* is one of most widely used bacteria to produce the recombinant proteins. It is cheap and fast-growing. As a prokaryotic system, however, the protein production lacks proper post-translational modification and may contain endotoxin, which is a major concern for food and pharmaceutics industries. Mammalian cell culture provides the best condition for generating protein with its high homology with living animal and fine machinery for protein processing and secretion. However, most of the mammalian cells have strict culture requirements and can be only cultured in attached surface and the yield is low while has the highest cost compared to other methods.

Yeast and *Dunaliella* are both eukaryotic and unicellular organisms. They can provide eukaryotic protein processing and achieve high turbidity of cell growth, which means higher recombinant protein productivity than the mammalian cell line expression system. Compared to the yeast expression systems, *Dunaliella* have caught the eye of industries in recent years with their special qualities and several advantages in producing proteins (Barzegari, Hejazi et al. 2010; Feng, Li et al. 2014).

First of all, *Dunaliella* are extremely halotolerant. They can grow from 0.2% salt concentration to 35% salt saturation concentration. In these cases, culturing *Dunaliella* in extreme salt concentration can easily eliminate contamination of other microalgae or bacteria and reduce the occurrence of pathogens. Secondly, *Dunaliella* are unicellular and contains no rigid cell wall. This feature would simplify the purification process. Thirdly, as photosynthetic organisms, the media for culturing *Dunaliella* is simple and cheap. For example, sea water can be used for the culturing of marine species. Lastly, *Dunaliella* accumulate large amount of by-products such as β - carotene, lipids and glycerol within the cells which will extend the industrial value of *Dunaliella* (Tafreshi and Shariati 2009).

As a novel host, *Dunaliella* have attracted increasing research attention. Exogenous genes have been successfully expressed in *D. salina* host by genetic engineering, such as the hepatitis B surface antigen (Geng, Wang et al. 2003; Geng, Han et al. 2004). However, big problems still

exist in using *Dunaliella* expression system, for example, low transformation efficiency and abundant time consumption after transformation in selecting successful transformants.

Table 2-2: Successfully used gene markers for selection of *D. salina* transformants

Name	Description	Annotation	References
Bar	Phosphinothricin acetyltransferase (bialaphos resistance gene)	Selective marker (phosphinothricin, PPT)	(Jiang, Lu et al. 2005)
Ble	Bleomycin (Zeocin) resistance gene	Selective marker (zeocin)	(Sun, Yang et al. 2005)
CAT	Chloramphenicol acetyltransferase	Selective marker (chloramphenicol) or reporter gene	(Wang, Xue et al. 2007)
EGFP (GFP)	(Enhanced) green fluorescent protein	Reporter gene	(Li, Han et al. 2011)
GUS	β-Glucuronidase	Reporter gene	(Geng, Han et al. 2004)

Up to now, several genes have been successfully transformed into *D. salina* as selective markers after transformation (Table 2-2). However, most selective markers such as GUS reporter gene were expressed transiently, and the antibiotic resistance ability, such as zeocin resistance would decrease after first and second sub-culturing (Sun, Yang et al. 2005). Therefore, in some cases, binary vectors were constructed with both a reporter gene and a selection marker in *Dunaliella* transformation system. For example, the pBI221-bar plasmid

(Feng, Xue et al. 2009) used in *D. salina* transformation consist of both a *bar* and a *GUS* gene.

In a recent work, different transformation methods have been summarized (Feng, Li et al. 2014). *Dunaliella* transformation methods via electroporation, particle bombardment, glass beads, and lithium acetate/polyethylene glycol (LiAc/PEG)-mediated method were compared. Among them, electroporation and particle bombardment have only 2% and less than 1% transformation rate, and both methods requires specialized equipment, namely high experimental expenses. Glass beads and LiAc/PEG-mediated methods have low physical damages to *Dunaliella* cells, but the highest transformation rates are only 5.9% for glass beads and 7.21% for LiAc/PEG-mediated method. Besides the above mentioned methods, *Agrobacterium* mediated method, a powerful transformation method widely used for plants (Zheng, Liu et al. 2012), has been explored for the efficient green algal transformation. It is reported that its transformation frequency was 50-fold higher than that of the glass beads transformation to the nuclear genome of the green alga *Chlamydomonas reinhardtii* (Kumar, Misquitta et al. 2004). Successful use of this *Agrobacterium* mediated transformation method has also been reported in other green microalgae such as *Haematococcus pluvialis* (Kathiresan, Chandrashekar et al. 2009). The simple and high transformation frequency, as well as the stable nuclear integration and expression of transgenes in those transgenic green algae reveal that the *Agrobacterium* mediated transformation shall be an efficient method for

the gene transformation of *Dunaliella*, and will enable further molecular study of *Dunaliella* to understand its basic metabolic processes as well as to exploit transgenic *Dunaliella* strains for biotechnological applications.

2.4.3 Genetically engineered *Dunaliella*: future candidates for solar energy utilization and CO_2 assimilation

Production of biofuels and valuable bio-products in the autotrophic *Dunaliella* is coupled with solar energy utilization and CO_2 mitigation (Araújo, Gobbi et al. 2009). In the microalgal production system, photosynthetic productivity and light utilization efficiency of *Dunaliella* are very important factors in the determination of cost. *Dunaliella* biomass production from natural salt water and CO_2 has been studied (Cortiñas, Silva et al. 1984). Laboratory experiments have also been conducted to evaluate the physical factors such as temperature and light intensity that could be enhance the photosynthetic biomass production by *Dunaliella* (Brown and Gasanov 1974; Sosik and Mitchell 1994).

On the other hand, the successful gene transformation methods enable the genetic engineering of the green microalgae *Dunaliella* to improve their energy production phenotypes. A foreign gene, *Chlamydomonas reinhardtii sedoheptulose-1,7-bisphosphatase* in the Calvin cycle, has been expressed in *D. bardawil* and improved photosynthetic performance was reported along with increased total organic carbon content and glycerol production (Fang, Lin et al. 2012). It demonstrated

the potential of using genetically engineered *Dunaliella* to increase and modify the accumulation of glycerol, lipids, total biomass, and other energy storage compounds.

Besides the specific gene targeted modification of *Dunaliella*, random mutagenesis by DNA insertion or chemicals has been performed on *D. salina* (Polle, Kanakagiri et al. 2002). It was found that a truncated chlorophyll antenna size is important in terms of the photosynthetic productivity of a mass culture, and a list of genes that confer a truncated light-harvesting chlorophyll antenna size to green algae was compiled. It is plausible to use these molecular and genetic approaches in future applications of *Dunaliella* utilizing CO_2 and wastewater, for example, genetically modified *Dunaliella* strain with enhanced photosynthetic productivity and light conversion efficiencies, and higher biofuel production rate.

2.5 Osmo-regulation in *Dunaliella*

2.5.1 Compatible solute and osmo-regulation

Early studies on halophilic organisms (Aitken and Brown 1972; Brown and Simpson 1972), as well as *Dunaliella* (Brown 1990), used the term “compatible solute” to describe the intracellular solute that the organism produced at high osmolarity to ensure the cell growth capability. Compatible solute is normally low molecular weight and can be accumulated till a high intracellular concentration. This “compatible solute” also helps maintain enzyme activity under hyper-saline

environment, and cells use such compatible solute as the osmolyte to achieve osmotic balance status across the cell membrane in osmo-regulatory mechanisms.

Potassium (K^+ or KCl) in halophilic bacteria was the only reported inorganic osmolyte (Larsen 1969, Aitken et al 1970, Aitken and Brown 1972,). Compared to the inorganic osmolyte, organic compatible solutes, such as glycerol and amino acids, are more common and efficient osmo-regulators in most halophiles, as organic compounds are usually uncharged at physiological pH, and have little inhibitory effects on enzyme activities but high solubility in the intracellular matrix. For example, glycerol in yeast or *Dunaliella* has high solubility and no net charge. It contains no aliphatic chain, and has very little affinity for the macromolecules and in addition, its effect on water structure is very small.

The mechanism by which an organism adapts to various salt concentrations has been termed “osmo-regulation” (Ben-Amotz, Shaish et al. 1991). *Dunaliella* is a good model for the investigation of salt-tolerant mechanisms and osmo-regulation because of their advantages in comparison to higher plants:

1) *Dunaliella* species are less differentiated than higher plants and the osmo-regulation occurs at individual cellular level;

2) *D. tertiolecta* is fast growing, which significantly reduces the study time compared to land grown plants;

3) The water potential of *D. tertiolecta* cell environment is relatively constant, in contrast to the cells of higher plants.

2.5.2 Glycerol serves as an osmolyte in *Dunaliella*

The genus *Dunaliella* includes a variety of widely distributed species. They have been found in differing habitats ranging from fresh water to saturated NaCl solutions. Lacking of a rigid cell wall (Hoshaw and Maluf 1981) provides for cell membrane elasticity, permitting rapid changes in cell volume in response to osmotic pressure. Osmo-regulation in *Dunaliella* depends on the regulation on glycerol production, which is the major intracellular osmolyte.

Glycerol is produced by *Dunaliella* as an important osmolyte to maintain osmotic balance in high salt environments. However, indications for this osmo-regulatory role of glycerol were not published until in 1964 (Craigie and McLachlan 1964). Later, other studies (Ben-Amotz and Avron 1973) established the function of glycerol in *D. parva* by using ^{14}C-bicarbonate and light photosynthetic bio-fixation. After that, the role of glycerol was further confirmed in *Dunaliella* salt adaptation process (Borowitzka, Kessly et al. 1977). *D. parva* accumulated very large amounts of intracellular glycerol and that the intracellular concentration of glycerol was osmotically equivalent to external NaCl concentration (Ben-Amotz and Avron 1973), thus it was obvious that glycerol served as the major osmotic component in the halophilic alga *D. parva*. Similarly, a linear relation between salinity and glycerol content was observed in *D. parva* over a broad range of salt

concentrations. This made it clear that glycerol was an important internal osmolyte to maintain the osmotic balance. Thus, glycerol formation and degradation in *Dunaliella* is the osmo-regulatory mechanism maintaining suitable osmotic pressure within the cells.

In Ben-Amotz and Avron's study, no leakage of intracellular glycerol was observed although intracellular glycerol could rapidly vary accordingly in adaption to hypo- and hyper-osmotic stress environments, when the extracellular salt concentration was increased or decreased (Ben-Amotz and Avron 1973). These alterations in glycerol content are interpreted as due to metabolic formation and degradation of intracellular glycerol. This further indicates the osmo-regulation pattern of *Dunaliella*, in which the osmotic balance depends on the variation of intracellular glycerol concentrations by glycerol synthesis or degradation in response to the external salinity. This dynamic osmo-regulation on glycerol content has been further demonstrated by Goyal (Goyal 2007; Goyal 2007). After studying the flux of the carbon fixed in photosynthetic process and that stored in starch in cells, they found that both glycerol synthesis and dissimilation occurred in cells at any salinity (osmotic pressure), and a hyper-osmotic salt stress could stimulate the glycerol synthesis significantly.

Both Ben-Amotz and Avron's and Goyal's work gave indication of how glycerol is regulated in *Dunaliella.* Glycerol functions as the osmotic counterweight providing a relatively suitable environment for *Dunaliella* to grow. Osmo-regulation in *Dunaliella* involves the synthesis of large

amounts of intracellular glycerol which serves to balance the external osmotic pressure. The variation of glycerol content as well as cell size in response to osmotic shock was summarized in details (Chen and Jiang 2009). Generally, osmotically-treated *Dunaliella* cells rapidly change their cell shape and volume, and maintain osmotic balance by synthesizing and varying the intracellular concentrations of glycerol, as shown in Figure 2-4. Extreme hypo-tonic osmotic conditions would result in cell burst while under extreme hyper-tonic conditions (high salt concentrations greater than 4 M), *Dunaliella* cells were observed to lose their flagella and build up a thick surrounding mucous to round the cell (Loeblich 1969).

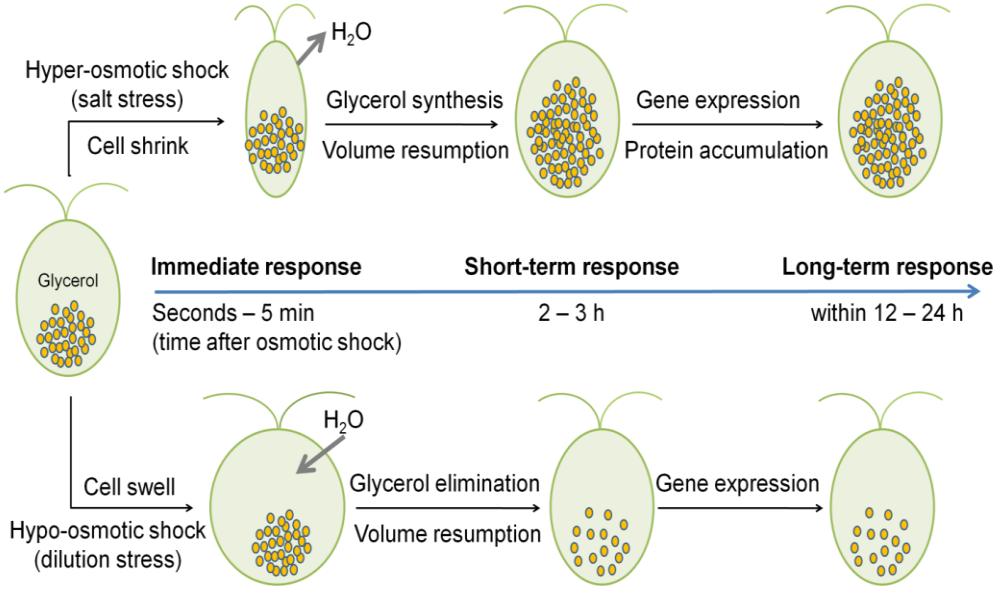

Figure 2-4 Osmo-response of cell morphology and glycerol production in *D. salina*

2.5.3 Photosynthetic behavior and the carbon flux to glycerol under osmotic stress

The photosynthesis can be affected by many factors such as temperature, light intensity, carbon dioxide, and the salt stress.

Photosynthetic microalgae cells are primary producer in the nature. Halotolerant species of *Dunaliella* are challenged by the hyper-saline environments both ecologically and metabolically. Salinity is a major limiting factor for growth of plants by inhibiting their central metabolic activities, especially the photosynthesis (Giordano and Beardall 2009).

Dunaliella is considered as a model photosynthetic halotolerant organism as they can adapt to high salinity environments. Salinity stress leads to a series of changes in its photosynthesis. A general model for photosynthetic performance has been established based on experimental study of the photosynthetic behavior of *D. parva* at three different NaCl concentrations (1 M, 2 M and 3 M) (Jiménez, Niell et al. 1990). It was found that *D. parva* showed the highest photosynthetic rates at 2 M NaCl, which is a relatively high salinity for most green plant species. This gives a good evidence that *D. parva* is a halotolerant species as its photosynthesis rate was still very high in such a salt stress.

Moreover, *Dunaliella* cells respond to high osmolarity by increasing free glycerol production. Glycerol has been shown to be a photosynthetic product (Craigie and McLachlan 1964), which presents a special case of photosynthetic behavior in response to osmotic pressure. It is remarkable that *Dunaliella* could enhance photosynthetic activity at high salinity (Liska, Shevchenko et al. 2004), since in most photosynthetic organisms such as plants and cyanobacteria, photosynthesis is inhibited by salt stress. By comparing the protein

profile of *D. salina* cells grown in low-salt (0.5 M NaCl) and high-salt (3 M NaCl) on two-dimensional gels, key enzymes in the Calvin cycle were identified to be induced by salt (Figure 2-5). Given the overall map of salt-activated carbon flux in *D. salina*, Figure 2-5 displays the direction of carbon flow leaded to glycerol synthesis, which indicated that *D. salina* responds to high salinity by diversion of carbon and energy resources for synthesis of glycerol, the osmolyte in *Dunaliella*. Therefore, it was suggested that energy metabolism and photosynthetic CO_2 assimilation of *D. salina* were enhances in 3 M NaCl compared to 0.5 M NaCl.

In *Dunaliella*, it was assumed that two different metabolic carbon flows were responsible for glycerol synthesis: one via a photosynthetic product and the other through the metabolic degradation of stored starch (Figure 2-5) and this presumption has been confirmed by studies of *D. salina* (Liska, Shevchenko et al. 2004) and *D. tertiolecta* wild types (Goyal 2007) as well as a *D. tertiolecta* salt-sensitive mutant (Goyal 2007). Goyal found that photosynthesis in *D. tertiolecta* was enhanced by mild salt stress, while inhibited at higher salt stress. Both photosynthesis and starch breakdown contribute carbon mainly in the form of dihydroxyacetone phosphate (DHAP) for glycerol synthesis. The relative contribution of each pathway appears to be dependent on the degree of salt stress.

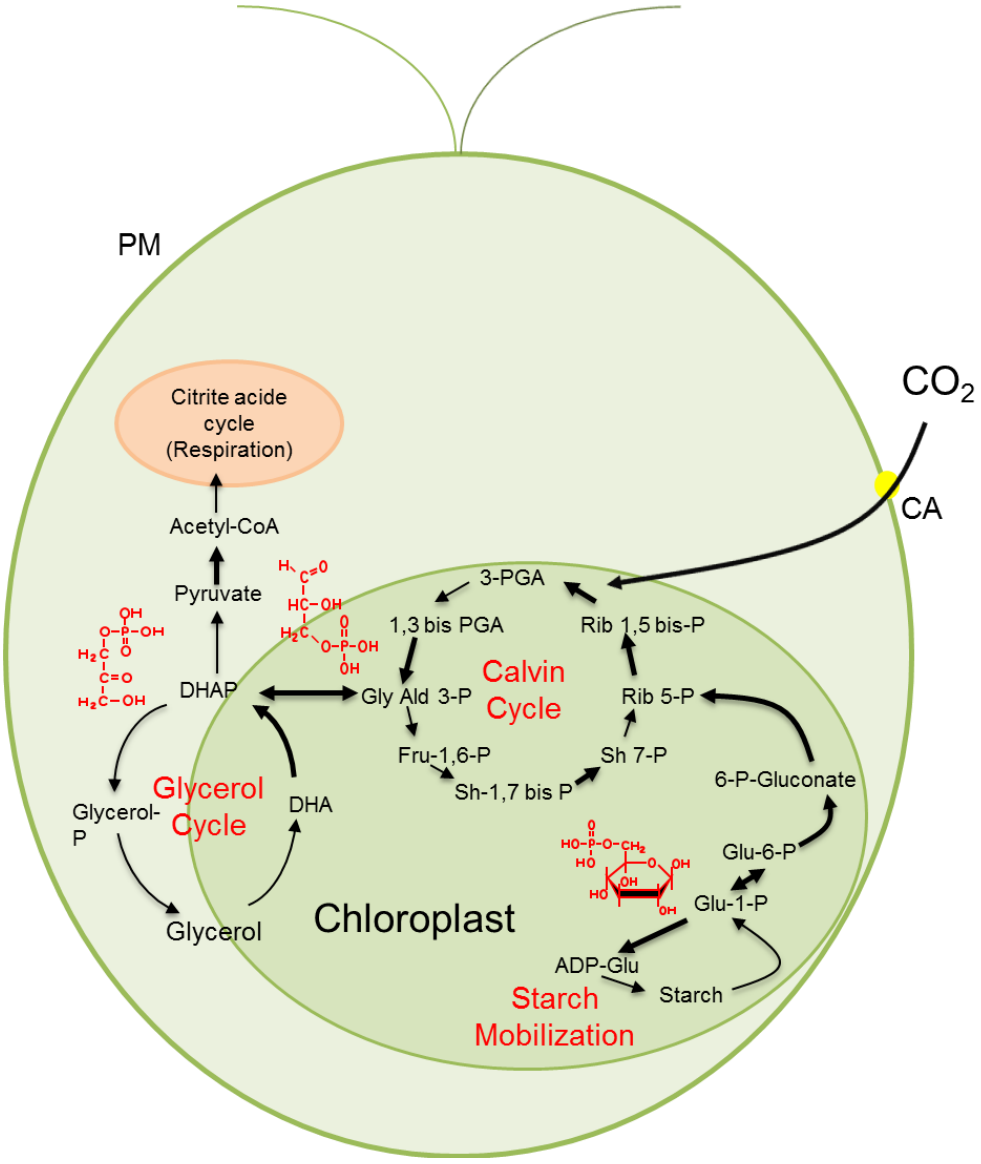

Figure 2-5 Metabolic pathways and salt-induced carbon flux to glycerol synthesis in *D. salina*
Thick arrows indicate the salt-induced enzymes identified in the work (Liska, Shevchenko et al. 2004). PM, Plasma membrane; CA, carbonic anhydrase.

2.5.4 Proteins up-regulated in osmo-response

The regulation of the response of plants to salt stress has previously been studied in model plant species with sequenced genomes, such as *Arabidopsis* (Consortium, 2000) and rice (Ning, Li et al. 2010). In yeast, the detailed High-Osmolarity-Glycerol (HOG) pathway, which regulates the cellular responses to the osmotic changes, is well studied now. In

Dunaliella, however, the detailed osmo-response at the molecular level is still unclear, mainly because the genome sequence of *Dunaliella* is unknown.

To date, molecular investigations on the specific enzymes as well as osmo-regulatory pathways involved in glycerol synthesis have been conducted at protein levels in *Dunaliella* (Avron 1992). Plasma membrane proteome study has suggested some specific membrane located proteins and enzymes to be induced by different salinities as an osmotic response of *Dunaliella*; most of those identified proteins were implicated in protein and membrane structure stabilization and located within signal transduction pathways (Katz, Waridel et al. 2007). For example, a 150 kDa membrane protein has been reported to be strongly induced by salt stress (Sadka, Himmelhoch et al. 1991), which was later identified as an unusual transferrin-like protein involved in the transport of iron into the cell (Fisher, Gokhman et al. 1997). Another noteworthy salt up-regulated membrane protein is the carbonic anhydrase, with an apparent molecular mass of 60 kDa (Fisher, Pick et al. 1994). It is said that the up-regulation of the plasma membrane located carbonic anhydrases facilitate CO_2 acquisition by *Dunaliella* (Fisher, Gokhman et al. 1996).

Recently, direct molecular approaches to study osmo-regulation in *Dunaliella* have been published reporting the encoding genes for GPDH enzyme in the glycerol cycle (Figure 2-3) of *D. salina* and *D. tertiolecta* (Ghoshal, Mach et al. 2002; He, Qiao et al. 2007; Yang, Cao

et al. 2007; He, Meng et al. 2009). Unique GPDH isoforms have been identified (Ghoshal, Mach et al. 2002; He, Qiao et al. 2007) with different properties than that of other plants and algae, which are responsible for the fast glycerol synthesis in *Dunaliella*.

Dunaliella have various specific proteins in response to changes in the medium salinity. To get more detailed information, protein profiles of *D. salina* cells grown at low-salt and high-salt conditions were compared on two-dimensional gels, and 76 proteins salt-induced by more than 2 folds were identified (Liska, Shevchenko et al. 2004). The proteomic analysis showed that most of these up-regulated proteins were enzymes of the central metabolic pathways involved in carbon assimilation and mobilization or in metabolic energy production, including the major Calvin cycle enzymes controlling photosynthetic carbon fixation, enzymes of the reductive pentose phosphate pathway/glycolysis, and a starch synthetic enzyme, as well as carbonic anhydrase located in the plasma membrane which enhances CO_2 uptake in *Dunaliella* at high salinity (Figure 2-5). This work was the first large-scale proteome analysis of salt up-regulated proteins in *Dunaliella*, a lower photosynthetic organism whose genome remains largely uncharacterized. The successful analysis and identification of the proteins has a significant impact on the study of the osmo-regulation in *Dunaliella*, as it can be used as a convenient model to study specific homologous proteins in response to external changes in salinity. However, only proteins up-regulated more than 2-fold were highlighted in this article, while minor protein effects were not described.

Some key factors might be missed in this analysis, for example, rate-limiting enzymes expressed in small quantities.

2.6 Potential osmo-regulatory mechanisms of *D. tertiolecta*

2.6.1 Metabolic pathways related to glycerol synthesis: glycolysis and pentose phosphate pathway

Dunaliella metabolic activities are regulated in response to osmotic stress. Among them, glycerol cycle is very important as the fixed CO_2 by photosynthesis is channeling into glycerol via this pathway, and thus new glycerol is synthesized. In addition to the photosynthetic CO_2, starch mobilizations under osmotic stress conditions (including glycolysis and its bypath pentose-phosphate pathway) also provide carbon for glycerol synthesis (Goyal 2007; Goyal 2007), suggesting that glycolysis and its bypath may be related to the osmo-regulation of glycerol synthesis in *D. tertiolecta*.

D. tertiolecta varies its glycerol contents rapidly in response to external osmotic stress change. Glycerol synthesis pathway, glycerolysis and the pentose phosphate pathway are involved in carbon mobilization during glycerol synthesis in *Dunlialla*. The majority of the intermediates required for glycerol synthesis generated by glycolysis (Figure 2-3), for example, dihydroxyacetone phosphate (DHAP), the precursor for glycerol synthesis (GIMMLER and MÖLLER 1981), is produced during glycolysis which occurs in the chloroplast (Figure 2-6). These pathways

are regulated by key enzymes, which modulate the flow of fixed carbon towards glycerol synthesis (GIMMLER and MÖLLER 1981; Chen and Jiang 2009). Glycolysis and the pentose phosphate pathway are important cellular metabolic pathways providing both energy (ATP and NAD(P)H) and substrates for other metabolic activities. Glycolysis and pentose phosphate pathway directly supply DHAP, the substrate for the glycerol cycle (Figure 2-6). It has been suggested that the oxidative stage of the pentose phosphate bypass may play a major role in keeping the redox balance during glycerol synthesis in *Dunaliella* (Chitlaru and Pick 1991). Besides, the crucial enzyme phosphofructokinase (PFK) in glycolysis pathway may be a checkpoint enzyme in the activation of glycerol synthesis, catalysing glucose to fructose-1, 6-diphosphate (Chen and Jiang 2009). In addition, a PFK encoding gene was found to be essential for glycerol accumulation in the budding yeast *Saccharomyces cerevisiae* during adaptation to high osmolarity stress (Dihazi, Kessler et al. 2004), and yeast cells over-expressing the *PFK* gene could accumulate 3 times more glycerol while cells lacking the same gene were not able to grow under hyper-tonic stress. This suggests PFK to be an important enzyme involved in regulating the glycerol synthesis.

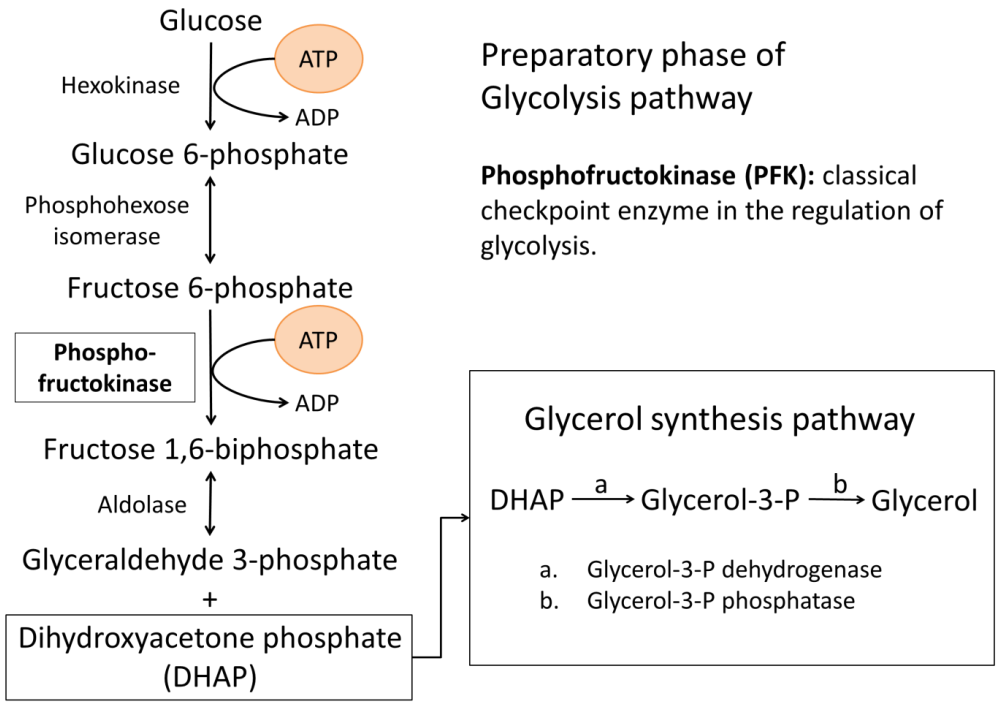

Figure 2-6 Glycolysis pathway (preparatory phase) provide the substrate DHAP for glycerol synthesis.

2.6.2 Other possible pathways: yeast HOG pathway

In addition to controlling at metabolic levels, glycerol production can be also regulated by other mechanisms. Similar to *Dunaliella* where glycerol is produced as the osmolyte to cope with the changing external osmotic pressure, glycerol production is also increased to prevent dehydration in the budding yeast *Saccharomyces cerevisiae* when exposed to hyper-osmotic stress. Researchers suggest that a signalling pathway is activated at the same time to regulate its glycerol synthesis and osmotic adaptation (Saito and Tatebayashi 2004; Hohmann 2009). This High-Osmolarity-Glycerol (HOG) signalling pathway is a Mitogen-activated-protein-kinase (MAPK) cascade (Figure 2-7). The HOG pathway is one of the well-studied multiple MAPK cascades in budding yeast. When stimulated by hyper-osmotic

challenge, the Hog1 protein leads to the expression of GPD1, which is the rate-limiting enzyme for glycerol synthesis, and thus results in increased intracellular concentration of glycerol as the counterbalancing osmolyte in yeast. The core molecule Hog1 in this pathway belongs to MAPK family, which is a highly conserved protein family present in all known eukaryotes and mainly involved in the regulating of cell stress responses.

MAPK-like proteins or genes have been studied in plants such as *Arabidopsis*, rice, maize, tobacco, as well as the green microalgae *Dunaliella*. In *D. salina*, a *MAPK* gene (*DsMPK*) was reported to be down regulated upon hyper-osmotic shock (Lei, Qiao et al. 2008). A MAPK-like protein (Jimenez, Berl et al. 2004) was suggested to be both up-regulated and phosphorylated in response to hyper-tonic shock in *D. viridis*. The protein could be recognized by antibodies specific to yeast Hog1 and its homologue mammalian p38. Other proteins with a phospho-p38 MAPK motif were found to be transiently phosphorylated after hyper-osmotic shock. Furthermore, inhibitors of MAPK activities could markedly impair the adaptation of *Dunaliella* to osmotic stress (Jimenez, Berl et al. 2004). All these investigations strongly suggest that MAPK cascade may be involved in the mechanism of osmotic regulation and a signaling pathway similar to yeast HOG pathway exists in *Dunaliella* to regulate the glycerol production in the cells.

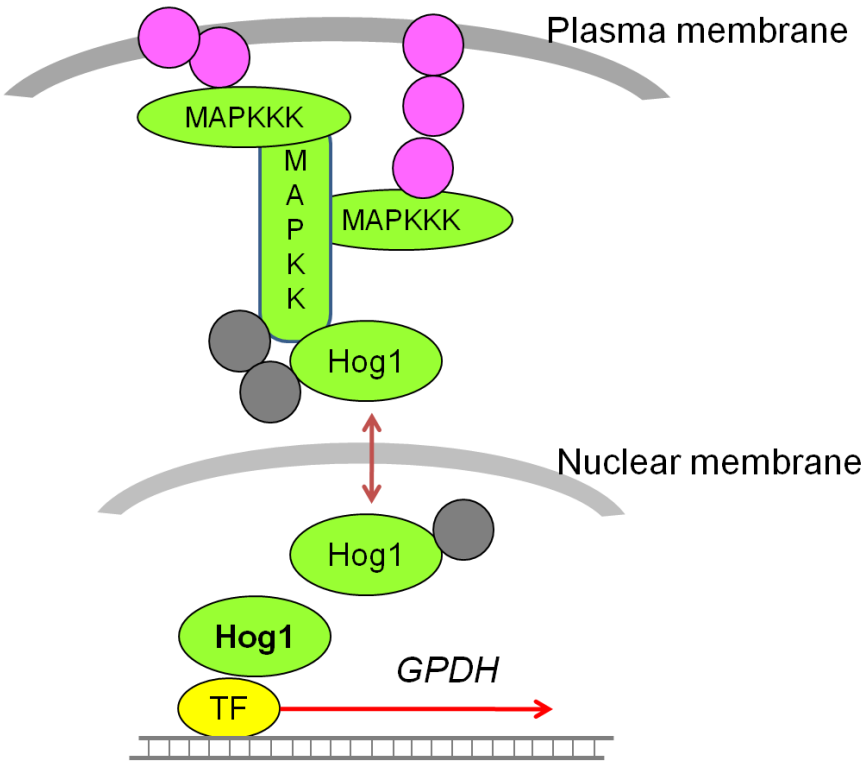

Figure 2-7 The yeast High-Osmolarity-Glycerol (HOG) pathway
Membrane-localised sensors and regulators are shown in purple, protein kinases including the mitogen-activated-protein kinase (MAPK) protein Hog1, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) in green, protein phosphatases in grey and transcription factor (TF) in yellow. Hog1 is the MAPK in this pathway, which accumulates in the nucleus under stress and induce the expression of *GPDH* for glycerol synthesis.

2.7 Summary

Global warming and the search for bio-fuels as green energy attract the attention to use *Dunaliella* as a candidate for CO_2 capture. Attempts have been made to exploit the bio-solar machinery to produce high concentrations of bio-products such as β - carotene and biomass for commercial use. Production of glycerol by *Dunaliella* has been shown technically possible in laboratory-scale, while its osmo-regulatory mechanisms for glycerol production have not been fully understood yet.

Significant progress has been made at the molecular level to address many questions related to the biochemical mechanism of osmo-

regulation in *Dunaliella*: Several key proteins involved have been identified in *Dunaliella*, provides us a preview of the mechanism type. However, major questions still remain to be addressed. For instance, what is the biochemical and molecular mechanisms that regulates the carbon flow to glycerol synthesis? What is the exact signaling pathway that triggers the osmo-regulatory responses? More studies are required before a satisfactory comprehension of the osmo-regulatory pathways is attained.

CHAPTER 3 PHYSIOLOGICAL STUDY OF THE OSMO-REGULATION IN *DUNALIELLA TERTIOLECTA*

3.1 Objectives and rationales

Dunaliella are desirable candidates in the establishment of green cell factories to assimilate atmospheric CO_2 and produce glycerol as an additional carbon sink. *Dunaliella* cultures have a variety of biotechnological applications such as production of the bioactive compounds, carotene, and glycerol.

So far a mostly well studied species from the genus *Dunaliella* is *D. salina*, due to its ability to accumulate >5% (up to 14%) of dry weight as β - carotene (Borowitzka and Siva 2007). *D. salina* cells have been successfully used in the commercial production of β - carotene (Del Campo, Garcia-Gonzalez et al. 2007; Kleinegris, Janssen et al. 2011). However, compared to this halophilic species which prefer an optimum salinity of about 20-25% NaCl (about 3.4-4.3 mol L^{-1}) (Borowitzka and Siva 2007). Saturated NaCl in water is about 6 mol L^{-1}. As a marine species, *D. tertiolecta* achieves optimal growth in media with much lower salinity (0.2-0.5 mol L^{-1} NaCl) (Jahnke and White 2003). Lower salinity required in culture media can significantly lower the cost for cultivation and corrosive nature of the media. Though *D. tertiolecta* does not produce as much β - carotene as *D. salina*, it has a much faster cell growth rate compared to other species such as *D. salina*

(Borowitzka, Kessly et al. 1977) and release glycerol from the cells, thus representing an efficient candidate for CO_2 mitigation with extracellular glycerol as an additional carbon sink for photosynthetically fixed CO_2 (Chow, Goh et al. 2013). Therefore, *D. tertiolecta* and its industrial and commercial potentials have been focused on.

Lack of rigid cell wall makes *Dunaliella* an excellent model to study stress signaling. Study on the biochemical and physiological response and the osmo-regulation mechanism of *Dunaliella* is of great interest. The morphology change and glycerol production pattern in response to hyper- or hypo-osmotic shock have been summarized in *D. salina* (Chen and Jiang 2009). While *D. tertiolecta* have significantly smaller cells than *D. salina* and its cell size has been considered less variable (Lin, Fang et al. 2013), it still remains unknown whether *D. tertiolecta* responses to osmotic shock exactly the same as *D. salina*. Since the regulatory mechanisms for glycerol production by *D. tertiolecta* have not been completely studied, this project started with the cellular osmotic responses of *D. tertiolecta*, including gross structural and metabolic changes at different osmotic stress conditions. The structural changes were described by the variation of the cells size while the metabolic changes were described by metabolic activities involved in the carbon flux from CO_2 and starch to glycerol.

3.2 Materials and Methods

3.2.1 The algal strain and culture conditions

Dunaliella tertiolecta LB-999 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). *D. tertiolecta* cells were grown in batch system in flasks, in the sterile modified ATCC-1174 DA liquid basal medium (Table 3-1) with various NaCl concentrations (0.5 M, 1 M, 2 M, 3 M and 4 M were used in this study), agitated by a rotary shaker and incubated at 25 °C, under a 14 h Light/10 h Dark photoperiod of approximately 50 µmol photons m^{-2} s^{-1} fluorescent light. Irradiance was measured using a Model LI-189 Quantum Meter (LI-COR, USA).

Table 3-1: Components of the modified ATCC 1174 DA basal medium

Compound	Concentration
$NaHCO_3$	0.02 M
Tris-HCl (pH 7.5)	0.05 M
KNO_3	5 mM
$MgSO_4$	0.01 M
$CaCl_2$	0.3 mM
KH_2PO_4	0.1 mM
FeCl3 dissolved in 20 µM EDTA	2 µM
H_3BO_3	100 µM
$MnCl_2$	0.5 µM
$ZnCl_2$	0.1 µM
$CuCl_2$	0.2 nM
$CoCl_2$	20 nM

3.2.2 Cell density and division rate

For growth and maintenance of cultures, *D. tertiolecta* cells were inoculated into fresh media at a 10% v/v ratio in a 250 mL screw capped conical flasks with continuous shaking (100 rpm). Cell density was monitored by microscopy counting using a hemacytometer (Neubauer Improved Grid) loaded with 10 µL of cell culture. Before counting, cells were fixed with 5% v/v of the 2% (v/v) formaldehyde solution. Three repeat counts were performed on triplicate samples.

Specific division rates of the algae cells (cell growth rate) were determined as previously described (Rao 2009). *D. tertiolecta* cell density was measured every day in order to plot the growth curve. All the growth curves were repeated at least three times. Specific division rates (μ) were calculated from the slope of logarithmic phase according to the formula (Equation 3-1).

Equation 3-1 Cell division rate formula

$$\mu = \frac{ln(n_2 / n_1)}{t_2 - t_1}$$

Where n_1 and n_2 represent cell numbers at times t_1 and t_2 in logarithmic phase ($t_2 > t_1$).

3.2.3 Treatments of hyper- or hypo-osmotic shock

D. tertiolecta cells were first grown in sterile 2 M NaCl ATCC-1174 DA liquid medium as previously described under photo-cycle illumination for 5 days (logarithmic phase). The *D. tertiolecta* cells were harvested

by centrifugation at 5000 × g, 10 min and 4 °C. Then the microalgae pellets were respectively suspended in isovolumetric experimental media with 4 M NaCl for hyper-osmotic shock or with 0.5 M NaCl for hypo-osmotic shock, and 2 M NaCl for isosmotic treatment. Treated *D. tertiolecta* cells were harvested accordingly at 0.5 h, 1 h, 2 h, 4 h, 6 h and 8 h (short-term osmotic shock) or 5 days (long-term osmotic shock) by centrifugation at 5000 × g, 10 min and 4 °C. The cell pellets were either frozen in liquid nitrogen and kept at -80 °C, or subjected immediately to subsequent steps. Each osmotic shock treatment experiment was repeated three times.

3.2.4 Determination of glycerol concentration

The extracellular and intracellular glycerol were extracted and assayed to determine the glycerol production. One mL *D. tertiolecta* liquid culture was centrifuged at 5000 × g at 4 °C for 10 minutes and the supernatant was directly used for assay of extracellular glycerol. The cell pellet was washed with fresh medium once and resuspended into distilled water with a final volume of 200 µL. After vigorous vortexing the suspension was boiled for 10 min and then centrifuged at 12,000 × g for 5 minutes to precipitate the cell debris. The supernatant was used for intracellular glycerol assay. Glycerol concentration was determined using the Free Glycerol Determination Kit (FG0100, Sigma-Aldrich, St Louis, MO). In addition to the instructions provided by the manufacturer, the reagent and sample volumes were scaled down 5 times. The sample-reagent mixture was incubated in 96-well plates for 15 min at

room temperature, and analyzed using a microplate reader (Tecan Infinite® 200 PRO) at 540 nm. Experimental media were used as blanks.

3.2.5 Determination of starch content

D. tertiolecta cells at stationary phase were collected by centrifugation (5000 × g, 10 min, 4 °C). Cell pellets were then extracted with 10 mL 80% ethanol twice to remove glucose and maltodextrins, followed by determination of starch using Starch Assay Kit (STA20, Sigma USA), according to the instructions provided by the manufacturer.

3.2.6 Total chlorophyll extraction and determination

Logarithmic phase *D. tertiolecta* cells grown in ATCC-1174 DA medium containing different molarity of NaCl were fully spun down at 11000 × g for 15 minutes at 4 °C (sample volume is the Extraction volume, V_e). Chlorophyll was extracted using a mixture of DMSO and 90% acetone (1:1 v/v) to disrupt the cells (Filtered volume, V_f). Mixture was centrifuged at 5000 × g for 15 minutes and the supernatant was retained for absorbance measurements. Absorbance (A or A_o) values at 750, 664, 647, 630 nm were measured using a Genesys 10S UV-Vis UV spectrophotometer (Thermo Scientific). To correct for pheopigment, 40 µl of 1M HCl was added and the mixture was incubated at room temperature for 90 seconds before absorbance (A_a) was measured at 665 nm and 750 nm. Chlorophyll contents were calculated using the following trichromatic equations (Equation 3-2) and the modified

monochromatic equation (Equation 3-3) for pheopigment correction (Holm-Hansen and Riemann 1978; Lorenzen and Jeffrey 1980).

Equation 3-2 Trichromatic equations for chlorophyll caculation

$$Chlorophyll\ a = (11.85 \times (A_{664} - A_{750}) - 1.54 \times (A_{647} - A_{750}) - 0.08 \times (A_{630} - A_{750})) \times V_e / L \times V_f$$

$$Chlorophyll\ b = (-5.43 \times (A_{664} - A_{750}) + 21.03 \times (A_{647} - A_{750}) - 2.66 \times (A_{630} - A_{750})) \times V_e / L \times V_f$$

Equation 3-3 Monochromatic equation for pheopigment correction of chlorophyll a

$$Corrected\ chlorophyll\ a = 11.4 \times K \times ((A_{665o} - A_{750o}) - (A_{665a} - A_{750a})) \times V_e / L \times V_f$$

Where
L = Cuvette light-path in cm; 1 cm quartz cuvettes were used
V_e = Extraction volume in mL
V_f = Filtered volume in mL
R = 1.7 = Maximum absorbance ratio of A_{665o}/A_{665a} in the absence of pheopigments
K = R / (R-1) = 2.43
Concentrations are in µg mL^{-1}.

3.2.7 Determination of photosynthetic rate

Net O_2 evolution rates were measured with a Clark-type oxygen electrode (Rank Brothers Ltd) under a light intensity of 20 µmol photons m^{-2} s^{-1} at 25 °C. Freshly prepared isotonic medium was used to dilute the cell cultures to a 0.65 µg mL^{-1} chlorophyll final concentration. A 4 ml aliquot of the diluted cell suspension was transferred to the oxygen electrode chamber. Before measurement, 80

µl of 0.5 M sodium bicarbonate solution was added to the cell suspension before oxygen evolution measurements to ensure that oxygen evolution was not limited by the carbon source available to the cells. The rate of oxygen (O_2) evolution was recorded for a period of at least 3 min.

For the measurement of photosynthetic efficiency, the net O_2 evolution rates at 25 °C was measured at various irradiances (0-1200 µmol photons m^{-2} s^{-1}) with a similar process. Initial slope of the O_2 evolution rate (photosynthesis rate, *P*) versus light energy (*E*) was used to calculate the photosynthetic efficiency (*PE*) (Duarte and Rao 2009).

3.2.8 Cell size analysis and osmolarity calculation

Both the cell biovolume and cell surface area were determined to illustrate the cell size. For measurement of the cell sizes, the hyper- or hypo-osmotically shocked *D. tertiolecta* cells at each time points were photographed at 1000 times magnification under a light microscope. Cell length and width (a and b) were measured using the software ImageJ (ImageJ, US National Institutes of Health, Bethesda, MD, USA) (Russ 2010), and cell surface area (S) as well as cell biovolume (V) were calculated according to Equation 3-4, based on the geometric ellipsoid model (Sun and Liu 2003) shown in Figure 3-1. At least 30 representative cells for each sample were selected for cell size analysis.

After calculating the biovolume, the cellular osmotic pressure was estimated using the Morse Equation (Equation 3-5) derived by van't Hoff (Amiji and Sandmann 2003).

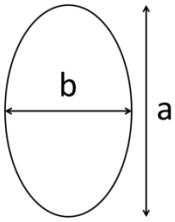

Figure 3-1 A prolate spheroid geometric model assumed for *D. tertiolecta* cell size measurement

Values of a and b were measured in the unit of µm using the software ImageJ, where a represents the major length of the ovoid cell model (length), and b represents the minor length of the ovoid cell model (width).

Equation 3-4 Equations for the calculation of cell biovolume and cell surface

$$V = \frac{\pi}{6} b^2 \, a$$

$$S = \frac{\pi \cdot b}{2}\left(b + \frac{a^2}{\sqrt{a^2 - b^2}} \, csc \, \frac{\sqrt{a^2 - b^2}}{a}\right)$$

Where V = cell biovolume (in the unit of μm^3) and S = cell surface area (in the unit of μm^2).

Equation 3-5 Morse Equation for the estimation of osmotic pressure

$$\Pi = n \, M \, R \, T$$

Where
Π = osmotic pressure
n = dimensionless van't Hoff factor = number of ions 1 molecule of solvent dissociates into
M = molarity of solute
R = gas constant (0.0821 L atm mol^{-1} K^{-1})
T = thermodynamic (absolute) temperature in kelvins (K)
The osmotic pressure Π was calculated in denomination of atm (standard atmosphere pressure).

3.2.9 Statistical analysis

Each result shown was the mean of three biological replicates, except for the cell size analysis where the mean of more than 30 representative cells for each sample were shown. The standard error of the mean (SEM) was calculated and presented as the error bar. Statistical analysis of the data was performed using the program SPSS-15, and significance was determined at a 95% confidence limit or with a p-value less than 0.05.

3.3 Results

3.3.1 Growth of *D. tertiolecta* under different osmotic stresses

Cell division rates of the logarithmic phase *D. tertiolecta* cells grown at different salinities were examined and shown in Figure 3-2. As can be seen, *D. tertiolecta* has a faster growth rate in media with lower salinities between 0.5 M to 2 M NaCl as compared to 3 M and 4 M NaCl. The optimal growth of *D. tertiolecta* was observed in salinity between 0.5 M and 2 M NaCl, which displayed similar division rates, while the growth rate was inhibited at high salinity environment. In spite of the observation that *D. tertiolecta* survived in 0.05 M NaCl ATCC-1174 DA medium, cells were found died in the second or third generation, hence the specific division rate were not calculated for *D. tertiolecta* at 0.05 M NaCl.

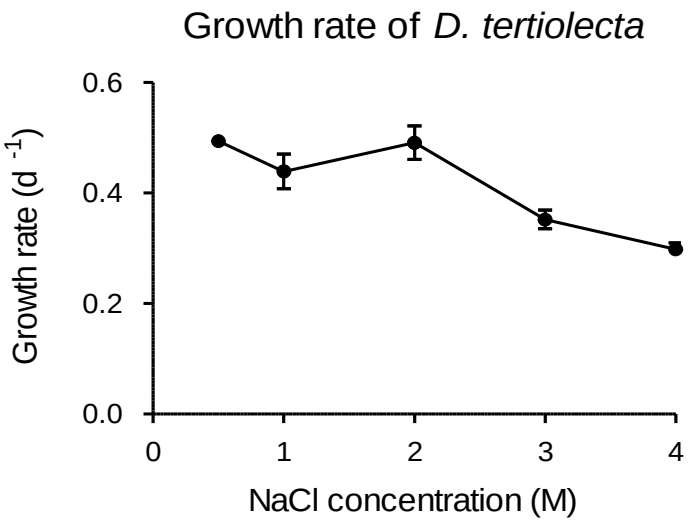

Figure 3-2 Cell growth rate of *D. tertiolecta* in different salt concentrations
Values shown are means of experimental triplicates. Division rates were calculated based on the logarithmic phase (first to fifth day after inoculation) growth curve of second generation cells. ATCC-1174 DA medium containing different concentrations of NaCl were used.

3.3.2 Glycerol production by *D. tertiolecta* under different osmotic stress conditions

In order to study how glycerol production by *Dunaliella* was regulated, glycerol content in *D. tertiolecta* cells was determined at different external osmotic stress conditions. Figure 3-3 showed both the intracellular and extracellular glycerol accumulated per cell over a period of 5 days after cells were transferred from 2 M NaCl to different salinities, ranging from 0.5 M to 4 M NaCl. The results suggest that glycerol production by *D. tertiolecta* was exactly proportional to the external salt concentrations. This finding further confirmed that glycerol is playing the important role in response to osmotic pressure in *D. tertiolecta*.

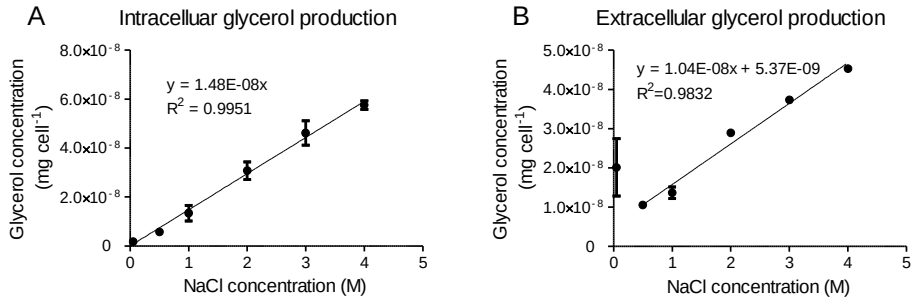

Figure 3-3 Glycerol accumulated by *D. tertiolecta* under various osmotic stresses

Values shown are means of experimental triplicates. Straight lines were generated by linear regression, and equations and R^2 were shown. A: Intracellular glycerol production; B: Extracellular glycerol production. Logarithmic phase of *D. tertiolecta* cells growing in 2 M NaCl ATCC-1174 DA medium were harvested and resuspended into ATCC-1174 DA medium containing 0.05 M, 0.5 M, 1 M, 2 M, 3 M or 4 M NaCl accordingly for different osmotic shock treatments. Glycerol concentrations were measured on the 5th day (120 h) after treatment.

Intracellular glycerol content of *D. tertiolecta* was regulated to achieve the osmolality balance across the cell membrane in media with different NaCl concentrations, while the extracellular glycerol also positively depending on the external salinity suggest that *D. tertiolecta* has higher total glycerol production at high salt conditions. The 0.05 M outlier of the extracellular glycerol production in Figure 3-3 B, where *D. tertiolecta* cells were shifted from medium containing 2 M NaCl to medium containing 0.05 M, might be caused by the cell burst due to the sudden decrease of osmolarity.

For a more detailed study of how glycerol production responses to osmotic shock, glycerol concentrations of *D. tertiolecta* were tracked at a series of time points for a period of 5 days after osmotic shock. Figure 3-4 shows the glycerol production by *D. tertiolecta* in response to both hypo- and hyperosmotic shock. *D. tertiolecta* could regulate its

glycerol production in response to different osmotic shock treatments. The short-term change of glycerol concentrations indicated that *D. tertiolecta* rapidly decrease or increase glycerol content to adapt to the external increase or decrease in osmotic stress (Figure 3-4 A and B). The intracellular glycerol concentration increased gradually from 1 h after a hyper-osmotic shock from medium containing 2 M to 4 M NaCl to cope with the increased osmotic pressure, while there was a sudden decrease of intracellular glycerol concentration at 0.5 h when cells were treated with hypo-osmotic shock from 2 M to 0.5 M NaCl, after which cells kept lowering the intracellular glycerol concentration gradually to reach a balance (Figure 3-4 A). The sudden decrease of intracellular glycerol content indicates that *D. tertiolecta* may have an emergency osmo-regulation on its glycerol content. During this emergency response process, large proportion of the intracellular glycerol leaked out into the media rapidly due to the sharp decrease of the salinity. This explanation could be further confirmed by the sudden increase at 0.5 h time point of extracellular glycerol concentration upon a hypo-osmotic shock (Figure 3-4 B).

The long-term response of glycerol production suggests that the intracellular glycerol concentration reached a steady level after cells had adapted to new osmotic stress, while the extracellular glycerol kept increasing for a long period of time (Figure 3-4 C and D). The steady level of intracellular glycerol content was expected as the cell needs to balance the osmolarity across the cell membrane. Intracellular glycerol concentrations were maintained at certain levels since 3 days after

treatment, which were roughly proportional to the salinity of medium (Figure 3-4 C). In the long-term response of extracellular glycerol production, faster glycerol synthesis rate was observed in higher salinity medium, although glycerol concentrations kept increasing at all conditions (Figure 3-4 D).

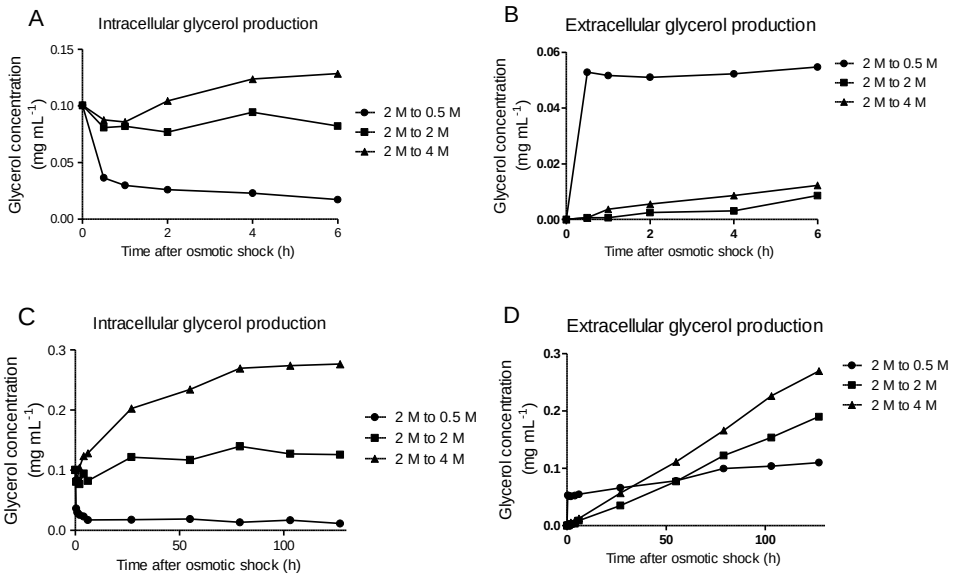

Figure 3-4 Glycerol production by *D. tertiolecta* in response to hypo- or hyper-osmotic shock
The intracellular glycerol concentration at time 0 was the glycerol concentration before osmotic shock treatment. Logarithmic phase of *D. tertiolecta* cells growing in 2 M NaCl ATCC-1174 DA medium were used. 2 M to 0.5 M (circle), cells were shifted to ATCC-1174 DA medium containing 0.5 M NaCl for treatment of hypo-osmotic shock; 2 M to 2 M (square), isotonic control; 2 M to 4 M (triangle), cells were shifted to ATCC-1174 DA medium containing 4 M NaCl for treatment of hyper-osmotic shock. A: Short-term response of intracellular glycerol content within 6 h after osmotic shock treatments. B: Short-term response of extracellular glycerol content within 6 h after treatment. C: Long-term response of intracellular glycerol production over 5 days (120 h) after treatment. D: Long-term response of extracellular glycerol concentration over 5 days (120 h) after treatment.

The intracellular glycerol in *D. tertiolecta* accounts for about 50% of the total intracellular osmolarity countering the culture medium osmotic stresses. The extracellular glycerol was continuously produced several

days after exposed to osmotic stress, with productivity positively related to salinity. At the same time, the intracellular glycerol remained fairly constant at a level required to maintain osmotic balance. It can be imagined that the large amount of extracellular glycerol is an important carbon sink for *D. tertiolecta*, since the external concentration of glycerol is not physically limited by cell size. Overall, the above results confirmed that glycerol production by *D. tertiolecta* was highly dependent on the medium osmolality, and cells could rapidly vary their glycerol content in response to sudden changes of external osmolarity.

3.3.3 Balance of osmotic pressures exerted by the medium salt and the intracellular glycerol in *D. tertiolecta*

The osmotic pressure was estimated based on the calculated cell biovolume at different salinities using Morse Equation (Equation 3-5). Figure 3-5 shows the osmotic pressure caused by the intracellular glycerol as well as the salt in external medium. As suggested in the figure, intracellular glycerol accounted for about 50% of the intracellular osmotic pressure countering the external osmotic pressure exterted by the saline medium. A linear relation was found between the osmotic pressures by intracellular glycerol and by salt in medium (Figure 3-6). Despite the intracellular glycerol pressure at 4 M NaCl in Figure 3-5 and Figure 3-6, it can be suggested that intracellular glycerol concentrations were dependent on the salinity of medium.

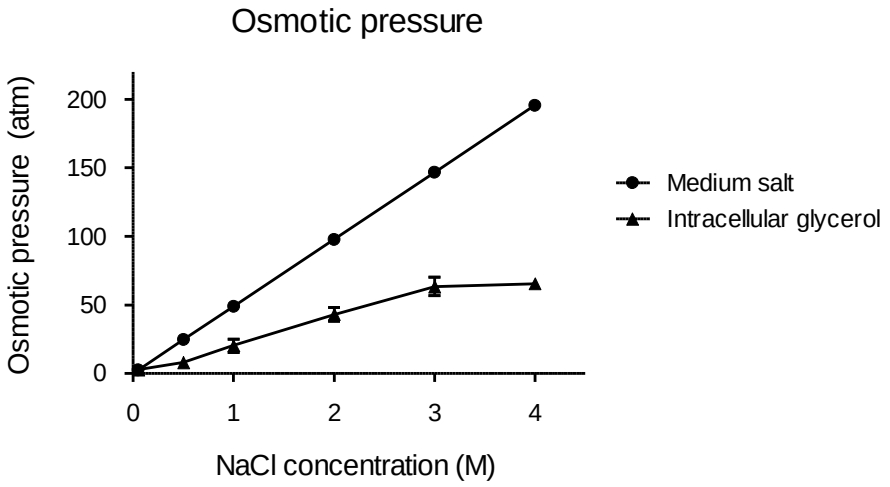

Figure 3-5 Osmotic pressures exerted by medium salt and intracellular glycerol in *D. tertiolecta*
Medium salt (circle): medium osmolarity; Intracellular glycerol (triangle): osmotic pressure exerted by intracellular glycerol. Early stationary phase (10 days old) of second generation cells grown in ATCC-1174 DA medium containing different molar of NaCl were used. For the calculation of intracellular glycerol osmotic pressure, the average biovolumes at different salinities (Figure 3-7 B) were used. Values shown are the means of three set of data based on the glycerol production in experimental triplicates.

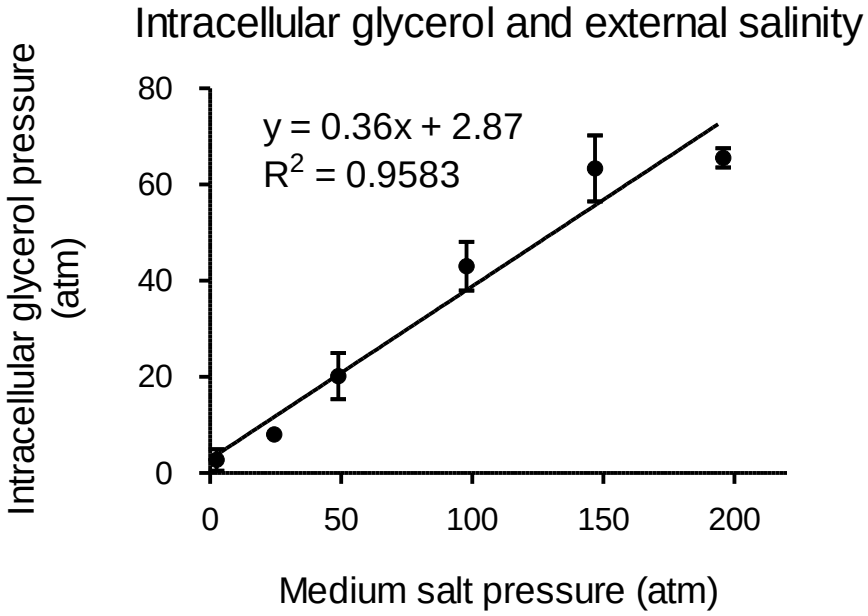

Figure 3-6 Relation between intracellular glycerol osmotic pressure and medium osmolarity
Straight line was generated by linear regression, and equation and R^2 were shown. Data used were the same as those in Figure 3-5.

3.3.4 Cell size variation under various osmotic stresses

Morphological characteristics of *D. tertiolecta* under different osmotic pressures were examined. Figure 3-7 shows the cell size of stationary phase *D. tertiolecta* cells grown in various salt concentrations. As a whole, cell sizes were closely correlated with extracellular salt concentrations. The smallest cell size was observed in medium containing 0.05 M NaCl and the largest cell size in 4 M NaCl medium. As intracellular glycerol accumulation (Figure 3-3 A) could be a major factor that affects cell size, it was reasonable to observe larger cell size in higher salinity. However, cell sizes in media with salinity ranging from 0.5 M to 2 M were comparable, although intracellular glycerol production was much higher in 2 M than 0.5 M. Given that *D. tertiolecta* has the fastest but similar growth rate (Figure 3-2) in the salinity range 0.5 – 2 M, cell size might be also affected by cell division. This reflects a complicated physiological osmo-response of *D. tertiolecta*.

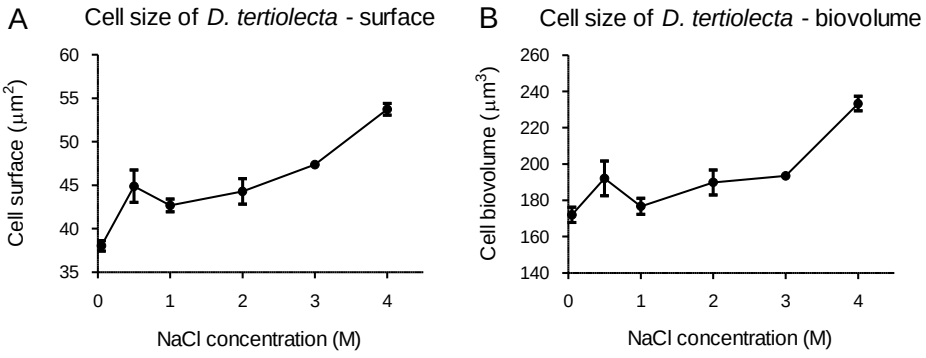

Figure 3-7 Cell size of the early stationary phase *D. tertiolecta* cells grown at different salinities

Values shown are the means of three independent experimental sets, and 30 cells were measured for each salinity in one experimental set. A: Cell surface area; B: Cell biovolume. Early stationary phase (10-day old) of second

generation cells grown in ATCC-1174 DA medium containing different molar of NaCl were used.

To explore the structural response of *D. tertiolecta* to changes in external osmolarity, the cell biovolume and cell surface area were determined to illustrate the variation of cell size. Responses of cell size to 2 M to 0.5 M hypo-osmotic shock or 2 M to 4 M hyper-osmotic shock are presented in Figure 3-8. As can be seen, cell surface and cell biovolume had similar responses. The cells size decreased upon hyper-osmotic shock due to cell shrinkage caused by loss of water arising from the concentration gradient across the membrane, while increased in response to hypo-osmotic shock by water flooding into cells. Figure 3-8 suggests that *D. tertiolecta* cell size could vary rapidly within 5-6 min upon osmotic shock, as change of cell surface or biovolume had already be seen since the first time point (0.1 h). The cell size of hyper-osmotic shock treated *D. tertiolecta* cells could gradually increase since 2 h after treatment (Figure 3-8B and D), when the intracellular glycerol also started to increase in the short-term response to hyper-osmotic shock (Figure 3-4 B), suggesting that the increase of cell size might be caused by intracellular glycerol accumulation. The larger cells upon hypo-osmotic shock gradually decreased until 8 h after treatment, where cells had a equal volume compared to the non-stressed cells (Figure 3-8A and C). The variation of non-stressed cell size could be caused by cell growth and division.

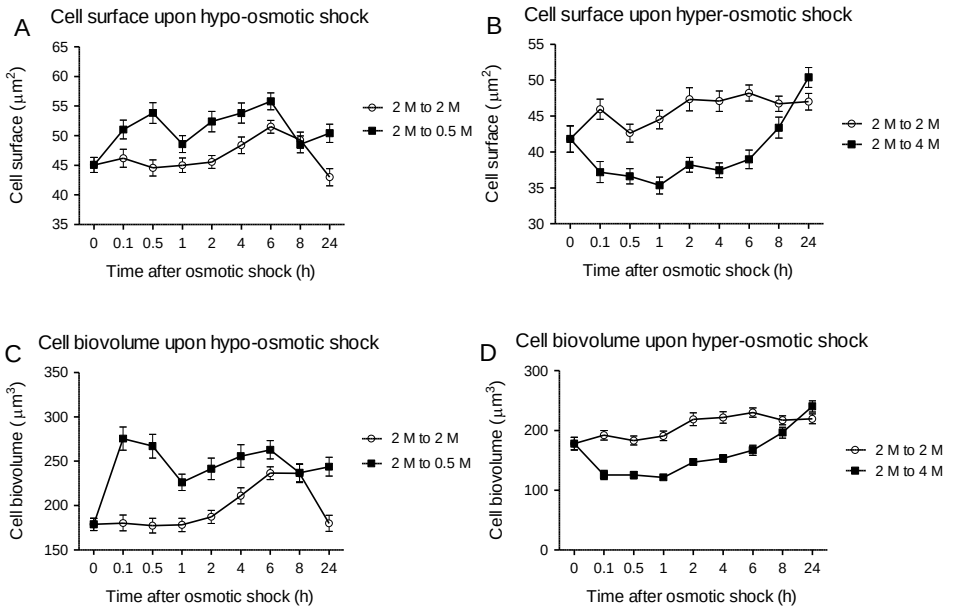

Figure 3-8 Cell size of *D. tertiolecta* in response to hypo- or hyper-osmotic shock

D. tertiolecta cell surface (A and B) and cell biovolume (C and D) were measured in response to hypo-osmotic shock (2 M to 0.5 M), hyper-osmotic shock (2 M to 4M) and the isotonic control (2 M to 2 M). The cell surface or biovolume at time 0 was the cell size measured before osmotic shock treatment. Values shown are the means of three independent experimental sets, and 30 cells were measured for each salinity in one experimental set.

3.3.5 Photosynthetic performance of *D. tertiolecta* in response to osmotic shock

The photosynthetic rate of *D. tertiolecta* in response to different osmotic shock treatments was investigated. In our study, rates of net oxygen evolution (nmol O_2 per 10^6 cells per second) were measured, and photosynthetic rate was calculated based on the initial slope of the plot of O_2 evolution rate versus time. Figure 3-9 shows the change of *D. tertiolecta* photosynthetic rate upon osmotic shock, over the initial 30 min or over a period of 90 min after treatment. The results suggest that osmotic shock could significantly affect the photosynthetic rate.

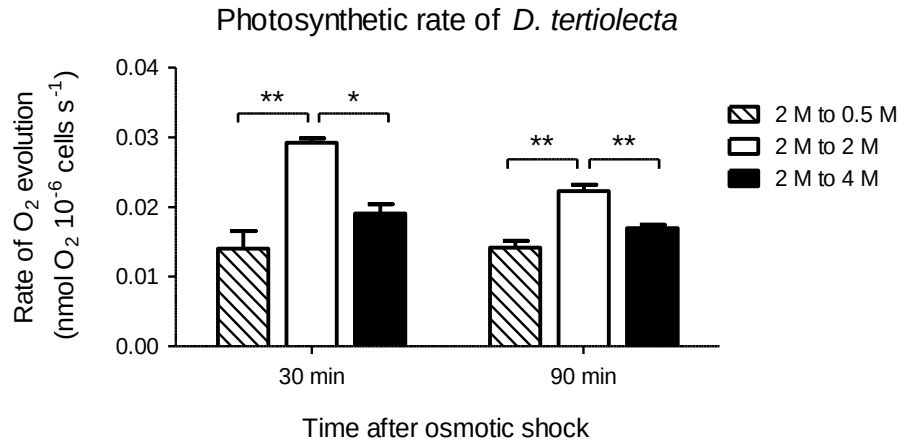

Figure 3-9 Photosynthesis rate of *D. tertiolecta* upon hypo- or hyper-osmotic shock
Logarithmic phase of *D. tertiolecta* cells growing in 2 M NaCl ATCC-1174 DA medium were used. 2 M to 0.5 M (shaded), cells were shifted to ATCC-1174 DA medium containing 0.5 M NaCl for treatment of hypo-osmotic shock; 2 M to 2 M (unshaded), isotonic control; 2 M to 4 M (black), cells were shifted to ATCC-1174 DA medium containing 4 M NaCl for treatment of hyper-osmotic shock. Photosynthetic rate was measured with the light intensity of 20 μmol photons m^{-2} s^{-1}. Values shown are the means of experimental triplicates.

D. tertiolecta cells adapted to the new osmotic condition within 24 hours after hyper-osmotic shock treatment, as suggested in Figure 3-4C, where the intracellular glycerol content has reached the steady level within 24 h in the long-term osmo-response study. In order to further confirm the suppression of photosynthesis under hyper-osmotic stress after the cells have adapted to the hyper-osmotic shock, the O_2 evolution rate of *D. tertiolecta* was tracked for a longer period of 5 days (120 h) and more detailed time points were taken within 24 hours. As shown in Figure 3-10, O_2 evolution rate of *D. tertiolecta* cells treated with a 2 M to 4 M NaCl hyper-osmotic shock were 50% lower than the control group for the entire period. It seems that photosynthesis was very sensitive to external salinity. This reduction in O_2 evolution rate

occurred within 15 min (0.25 h) in response to the hyper-osmotic shock treatment when shifted from 2 M to 4 M NaCl.

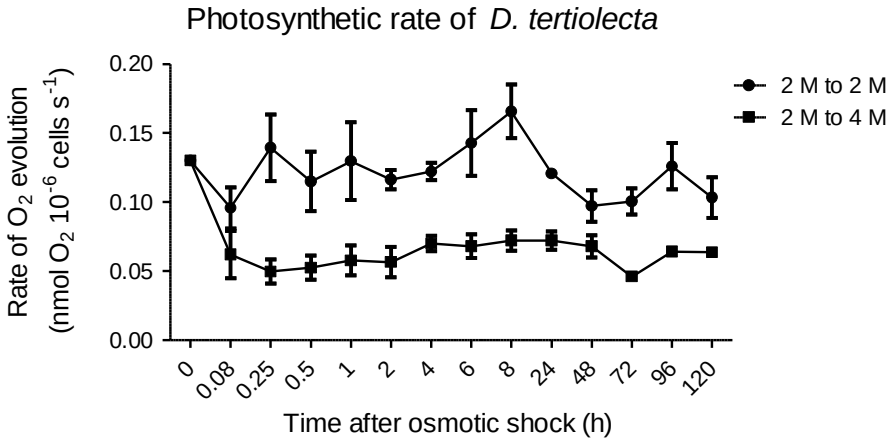

Figure 3-10 Photosynthetic rate of *D. tertiolecta* in response to hyper-osmotic shock from 2 M to 4 M
The photosynthesis at time 0 was the O_2 evolution rate of the cells before osmotic shock treatment. *D. tertiolecta* cells growing in 2 M NaCl ATCC-1174 DA medium were shifted to ATCC-1174 DA medium containing either 4 M NaCl for hyper-osmotic shock (square), or 2 M NaCl for isotonic control (circle). Photosynthetic rate was measured with the light intensity of 20 μmol photons m^{-2} s^{-1}. Values shown are the means of three independent experimental sets.

All the above information shows that O_2 evolution rate was decreased by raised salt concentration. To answer the question that whether this decrease is caused by the extremely high salinity (4 M), where photosynthesis activity is inhibited in most photosynthetic organisms, other hyper-osmotic shock treatments at lower salinities were applied to *D. tertiolecta*.

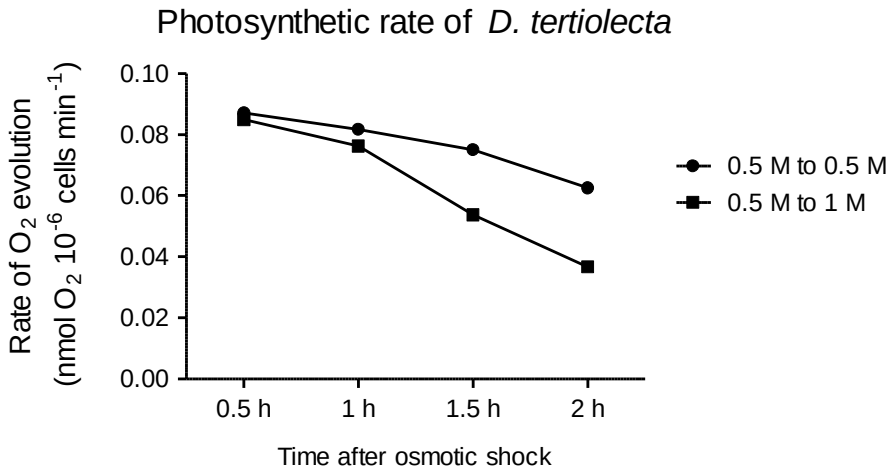

Figure 3-11 Photosynthetic rate of *D. tertiolecta* upon hyper-osmotic shock from 0.5 M to 1 M NaCl
D. tertiolecta cells growing in 0.5 M NaCl ATCC-1174 DA medium at logarithmic phase were shifted to ATCC-1174 DA medium containing either 1 M NaCl for hyper-osmotic shock (square), or 0.5 M NaCl for isotonic control (circle). Photosynthetic rate was measured with the light intensity of 20 μmol photons m^{-2} s^{-1}.

Figure 3-11 showed the osmo-response of O_2 evolution rate upon a hyper-osmotic shock at lower salinities from 0.5 M to 1 M NaCl. *D. tertiolecta* have optimal growth in this range of salinity (Figure 3-2), while the photosynthetic rate suggests that *D. tertiolecta* had decreased photosynthesis in response to hyper-osmotic shock. However, the O_2 evolution rate only started to decrease 1 h after the 0.5 M to 1 M hyper-osmotic shock, and progressively dropped to about half that of the control group at 2 h after treatment. Through the photosynthesis had slower responses than those cells under greater increase in osmolarity from 2 M to 4 M NaCl, where the rate decreased rapidly within 15 min, it still demonstrated that the suppression of photosynthesis was caused by hyper-osmotic shock.

Beside the osmo-response study of photosynthesis upon osmotic shock treatments, a profile of photosynthetic rate at different salinities was also investigated. O_2 evolution rates of the second generation of *D. tertiolecta* grown in ATCC-1174 DA medium containing different NaCl concentration was determined at the exponential phase. As *D. tertiolecta* cells have different chlorophyll content in different salinities, total chlorophyll concentrations per cell were measured before the experiment. The O2 evolution rate was calculated on a per cell as well as per chlorophyll (chl) basis, as shown in Figure 3-12. As can be seen, no clear trend was shown on the O2 evolution rate per 106 cells per second, while there was a progressive decrease with increasing salinity when considering the O2 evolution per second per µg of chlorophyll. It suggests a negative correlation between photosynthetic rate on a per chlorophyll basis and the external salinity. The photosynthetic efficiency would be inhibited by raising the salt concentration of the culture medium.

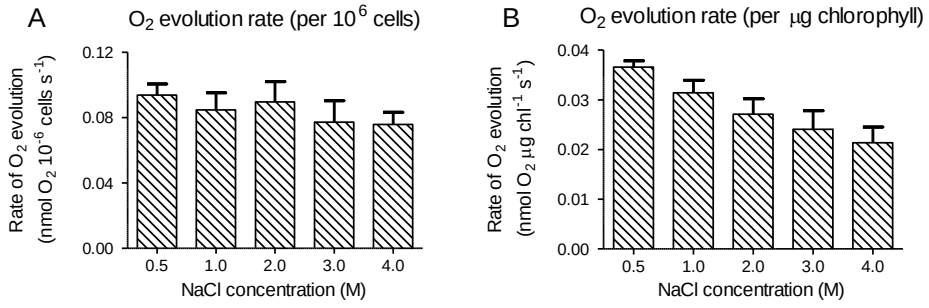

Figure 3-12 Photosynthetic rate of *D. tertiolecta* grown in different salinities
Logarithmic phase (5 days old) of second generation *D. tertiolecta* cells grown in ATCC-1174 DA medium containing different molar of NaCl were used. A: Photosynthetic rate plotted on the per 10^6 cells basis; B: Photosynthetic rate plotted on the per µg chlorophyll basis. Photosynthetic rate was measured with the light intensity of 20 µmol photons m^{-2} s^{-1}. Values shown are the means of three independent experimental sets.

3.3.6 Starch content of *D. tertiolecta* under different salinities

For further study of the carbon source for glycerol synthesis in *D. tertiolecta*, the starch stored by *D. tertiolecta* cells in different osmolarity conditions was determined and shown in Figure 3-13. As can be seen, *D. tertiolecta* has the highest starch storage of about 8 µg per 10^6 cells when growing in 0.5 M NaCl medium, where the cell has the optimal growth rate (Figure 3-2). However, *D. tertiolecta* has the lowest glycerol production in 0.5 M NaCl medium (except 0.05 M where cells could not survive for more than 3 generations) (Figure 3-3), with the intracellular glycerol at about 0.5 × 10^{-8} mg per cell and extracellular glycerol concentration at 1 × 10^{-8} mg per cell.

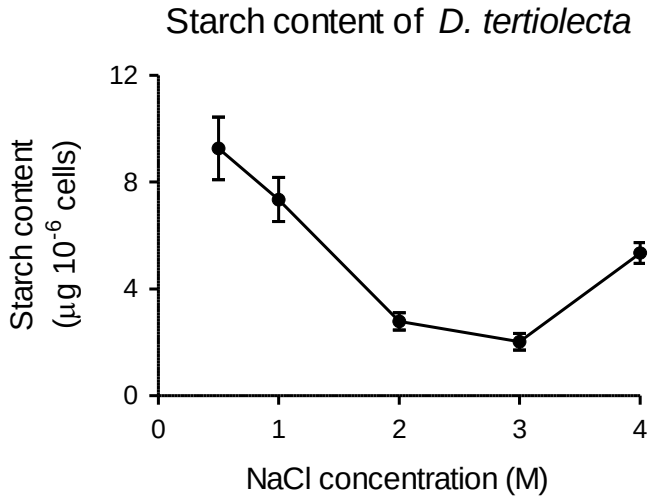

Figure 3-13 Starch content of *D. tertiolecta* grown in different salinities
Logarithmic phase (5 days old) of second generation cells grown in ATCC-1174 DA medium containing different molar of NaCl were used. Values shown are the means of three independent experimental sets.

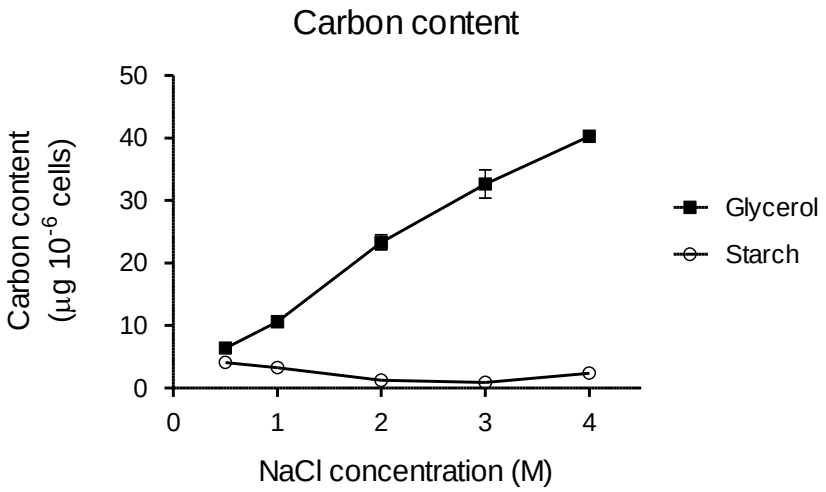

Figure 3-14 Carbon content of glycerol and starch accumulated in *D. tertiolecta*
Black square: carbon content in total glycerol produced (intracellular and extracellular glycerol); white circle: carbon weight of starch content. Logarithmic phase (5-day old) of second generation cells grown in ATCC-1174 DA medium containing different molar of NaCl were used. Values were calculated based on the data in Figure 3-3 and Figure 3-13.

To compare their relative amounts, cellular starch content as well as total glycerol production, including both intracellular and extracellular glycerol, was plotted in Figure 3-14 in the form of carbon weight. As shown in the figure, the carbon content of total glycerol was much higher than that of starch stored by *D. tertiolecta*, especially in 2 M to 4 M NaCl medium, where the glycerol carbon was 20-40 times higher than starch carbon. The low cellular starch content is imaginable as the cell size of *D. tertiolecta* is much smaller than *D. salina* under the same culture condition (Lin, Fang et al. 2013), which may apparently limit the starch storage (Liu, Ming et al. 2012). Despite the generally decreasing trend of starch content along with increasing salinity, carbon from starch degradation may only have a limited contribution to the highly increased glycerol production, and it is hard to reason that starch

degradation provides carbons for glycerol synthesis upon osmotic shock. Since cell growth was suppressed under high osmolarity (Figure 3-2), the reduction in specific growth rate could channel carbon meant for biomass growth to glycerol synthesis, thus an explanation for the high glycerol production by *D. tertiolecta* cells under 3 M and 4 M NaCl conditions.

3.4 Discussion

3.4.1 *D. tertiolecta* is a suitable candidate for industrial use

D. tertiolecta has the optimal growth in lower salinity compared to other halophilic *Dunaliella* species (Borowitzka and Siva 2007). Less salt required in the culture media will significantly reduce the corrosive nature of the media as well as the operating cost for cultivation. As sea water contains about 0.6 M salt, it was expected to observe this species growing faster in salinity ranging from 0.5 to 2 M NaCl. Even though glycerol production was proportional to external salinity from 0.5 M to 2 M salt conditions, it seems that growth of *D. tertiolecta* was not strictly sensitive to external salinity within this range, as growth rates had no significant difference among 0.5 M, 1 M and 2 M salt conditions.

The long-term osmotic stress treatment study suggested that *D. tertiolecta* kept synthesizing extracellular glycerol when intracellular glycerol was maintaining the osmotic balance. This finding is consistent with the reported osmo-responses of *D. salina* glycerol production (Chen and Jiang 2009). Salt stress as well as other stress conditions

have been applied to the cellular factory *D. salina* in order to increase the industrial production of β - carotene (Lamers, Janssen et al. 2008). Therefore, high salinity should be applied for increased productivity of glycerol by *D. tertiolecta*, despite that the growth rate are slightly lower at high salinity (Figure 3-2). Considering cell growth rate, 2 M NaCl condition may be a good choice as *D. tertiolecta* has fast growing speed as well as produces much glycerol at this condition. Industrial usage of *D. tertiolecta* in large scale cultivation can be achieved by controlling the osmolarity as well as other conditions of the media.

D. tertiolecta has been evaluated for use in carbon dioxide fixation through the production of microalgal biomass (Sydney, Sturm et al. 2010). Besides, *D. tertiolecta* had a high oil yield of 36 – 42% with high salinity (Chen, Tang et al. 2011), which makes *D. tertiolecta* another potential to be used for biodiesel production in large-scale outdoor cultivation. Therefore, *D. tertiolecta* is an important species with great industrially importance.

3.4.2 Extracellular glycerol as an additional carbon sink

D. tertiolecta continuously produce extracellular glycerol with higher producing rate in higher salinity. Hence extracellular glycerol is be an effective additional carbon sink for the assimilated carbon dioxide as it it not limited by cell volumes. *D. tertiolecta* displayed its potential to capture CO_2 in the atmosphere and synthesize glycerol at the same time. It has been demonstrated that *D. tertiolecta* produces much higher glycerol as compared to other carbon assimilation products such

as biomass (Chow, Goh et al. 2013). A technically feasible photosynthetic bioconversion process has been previously proposed for the production of intracellular glycerol from *Dunaliella* (Chen and Chi 1981). However, the economic feasibility was very low due to high capital and production costs. Process for large-scale *Dunaliella* glycerol production has not been developed so far (Oren 2005). It would be much useful to look into detailed investigation on bioprocess development for *Dunaliella* glycerol production.

3.4.3 Osmo-regulation in *D. tertiolecta*

The intracellular glycerol only counter-balanced about 50% of the culture medium osmotic pressure in *D. tertiolecta*. The lower intracellular osmotic pressure accounted for by glycerol as compared to the estimated osmotic pressure of intracellular glycerol required for osmotic balance at different salinity suggests that intracellular components other than glycerol may also contribute to maintain the osmotic balance across the cell membrane. The outlier from the linear trend of intracellular osmotic pressure and external medium osmotic pressure at 4 M NaCl also suggests that glycerol may not be the only osmolyte (Figure 3-6). There might be alternate osmo-regulatory mechanisms that function to maintain osmotic balance at high salinities. Glycine betaine is another osmolyte employed in some halotolerant bacteria. However, it has been demonstrated in a recent research work (Park, Kim et al. 2014) that the concentration of glycine betaine was very small as compared to glycerol and did not increase with salinity.

Hence, glycine betaine may not function as an osmolyte in *Dunaliella*. Another possible osmolyte may be the inorganic salt or ion, K^+ (or KCl) that contribute to the intracellular osmotic pressure in *Dunaliella*. The role of KCl was firstly identified in the halophilic bacteria *Halobacterium* (Larsen 1973) and further demonstrated as an osmolyte (Brown 1983; Larsen 2011). Researchers have also postulated that rapid cation exchange systems may operate as part of the osmo-regulatory system of *Dunaliella* in addition to glycerol accumulation (Latorella and Vadas 1973; Gimmler, Schirling et al. 1977; Katz and Pick 2001). *Dunaliella* controls ionic homeostasis in the cell through a specialized ion-pumping mechanism (Katz and Pick 2001). Cation such as K^+ may potentially function together with intracellular glycerol to regulate cellular osmotic balance. The ion-pumping mechanism might be also involved in the osmo-regulation in *Dunaliella*.

It has been known that *D. salina* could rapidly vary its cell volume and shape in addition to the regulation on glycerol concentration in response to osmotic shock treatments (Chen and Jiang 2009). Despite the fact that *D. tertiolecta* has much smaller cell size than *D. salina* (Lin, Fang et al. 2013), cells of *D. tertiolecta* could also vary cell volume and cell shape as an emergency osmo-regulation mechanism for the rapid response to external vary of Osmolarity. These morphological and structural responses are similar in both *D. tertiolecta* and *D. salina*. However, volume resumption of *D. tertiolecta* cells happened between 6-24 hours after osmotic shock (Figure 3-8C and D), which is much slower than the reported 2-3 hours for *D. salina* (Chen and Jiang 2009).

A hyper-osmotic shock treatment would cause a 50% decrease in cell volume in other *Dunaliella* species such as *D. salina* due to net water loss within a few minutes after treatment (Chitlaru and Pick 1989; Oren-Shamir, Pick et al. 1990). On the other hand, there was a much slower response in *D. tertiolecta* cell volume upon hyper-osmotic shock (Figure 3-8). This might be due to the less elastic cell membrane in *D. tertiolecta* (Lin, Fang et al. 2013). Therefore, there may be other mechanisms in the rapid osmo-response and recovery activities to counter a sudden increase in salinity changes before glycerol synthesis occurs.

3.4.4 Carbon source for glycerol production in *D. tertiolecta*

The carbon required for glycerol synthesis in *Dunaliella* derives from photosynthesis or by the degradation of intracellular starch (Goyal 2007; Goyal 2007). Photosynthetic rate and starch storage of *D. tertiolecta* were also studied in this project. It is interesting to find that *D. tertiolecta* displayed lower photosynthetic rates under both hypo- and hyper-osmotic shock conditions no matter that glycerol production was high or low. *D. tertiolecta* cells were stressed when the external osmolarity changed. This might be a reason for the significantly decreased O_2 evolution rate. The study on photosynthetic rate of *D. tertiolecta* will provide more information on its culture conditions in large-scale to assimilate CO_2 in the atmosphere and producing glycerol as the by product, i.e. osmolarity is suggested to be maintained steady in order to achieve high photosynthetic efficiency. As starch storage

content was negligible compared to glycerol, the carbon for glycerol synthesis provided by starch degradation should be very little, and starch may not be a major source of carbon for glycerol synthesis in *D. tertiolecta*. Therefore, reduction of cell growth under hyper-saline environment could be a possible reason for the high glycerol production by *D. tertiolecta*, where more photosynthetic carbon was channeled to glycerol synthesis instead of biomass growth.

D. tertiolecta displayed lower photosynthetic rates under both hypo- and hyper-osmotic stresses. However, photosynthesis of *D. tertiolecta* has been reported to be up-regulated by hyper-osmotic stress and down-regulated by hypo-osmotic stress (Goyal 2007; Goyal 2007): a 30% increase of the photosynthetic O_2 evolution was found when *D. tertiolecta* was stressed by raising the NaCl concentration, while the photosynthetic O_2 evolution was progressively decreased with decreasing the salinity. The result in this project shows that *D. tertiolecta* had lower O_2 evolution rate with decreasing salinity from 2 M to 0.5 M (Figure 3-9), which is in agreement with the previous reports (Goyal 2007; Goyal 2007). However, a 30% decrease was observed in the O_2 evolution rate of *D. tertiolecta* upon hyper-osmotic shock treatment (Figure 3-9), contrary to Goyal's reports. These interesting findings show the complicated response and regulation on the metabolic activities of *D. tertiolecta* cells upon various osmotic stresses.

It is known that photosynthetic efficiency in most plants is inhibited by high salt (Hasegawa, Bressan et al. 2000). Thus a decrease in the

photosynthetic rate upon hyper-osmotic shock was expected with increased external salinities. However, it is interesting to find that the photosynthesis was also inhibited when *D. tertiolecta* cells were treated with hypo-osmotic shock (Figure 3-9). Other researchers also reported that hypo-osmotic stress inhibited the photosynthesis efficiency in *D. salina* possibly through reactive oxygen species formation (Liu, Ming et al. 2012). This could be due to cell stress caused by the change in external osmolarity, such as a hypo- or hyper-osmotic stress condition, which make *D. tertiolecta* away from its optimum salinity for growth, thus the stressed *D. tertiolecta* cells resulted in a significantly decreased O_2 evolution rate. The findings hence provide information on the culture conditions when using large-scale *D. tertiolecta* to assimilate CO_2 and produce glycerol as well: Osmolarity of the culture media should not be changed in order to maintain the CO_2 fixation at a high level; Low salinity should be used since photosynthetic rate may be inhibited in high salt environment. However, *Dunaliella* glycerol production can be highly enhanced by high salinity. Thus the medium's osmolarity needs to be justified to achieve relatively high photosynthesis activity (faster fixation rate) as well as glycerol production (more carbon storage).

Along with the suppression of photosynthesis by increased salinity, more glycerol was synthesized to counter the increased extracellular osmotic pressure. These observations suggest that glycerol production was affected by factors other than photosynthesis, and there might be other carbon sources for *Dunaliella* glycerol synthesis in addition to the

photosynthetically fixed carbon at high salinity. Starch is a major carbon storage compound as a final product of photosynthesis in most green cells. Starch break-down has been suggested to be another important source of carbon for glycerol synthesis in some species of *Dunaliella* (Goyal, 2007a). Thus glycerol is produced via two possible routes in *Dunaliella* when exposed to external osmotic pressure: photosynthetic fixation of CO2, or by conversion of a reserve starch pool within the cell (Chow, Goh et al. 2013). The relative contributions of these two metabolic pathways may be different in different species of *Dunaliella* (Goyal 2007; Goyal 2007).

3.5 Conclusion

D. tertiolecta can grow in a wide range of salinities but prefers a relatively low salt environment such as sea water. the physiological responses to various osmotic stress conditions were evaluated In this chapter in order to investigate the osmo-regulatory mechanisms in *D. tertiolecta* for glycerol synthesis. *D. tertiolecta* showed similar responses to extracellular osmotic changes on glycerol regulation but some differences on cell size variation. It rapidly decreased or increased glycerol content to adapt to hypo-osmotic or hyper-osmotic shock. Besides, glycerol production by *D. tertiolecta* was proportional to the extracellular salt concentrations. In addition, *D. tertiolecta* can be used as an effective CO_2 scrubber as the large amount of extracellular glycerol production by *D. tertiolecta* is be an important additional

carbon sink. This study shed light on the commercial potential of using *D. tertiolecta* in large scale for glycerol production and CO_2 fixation.

CHAPTER 4 TRANSCRIPTIONAL STUDY OF *PFK* GENE INDICATED THAT STARCH MAY NOT CONTRIBUTE TO GLYCEROL PRODUCTION IN *DUNALIELLA TERTIOLECTA*

4.1 Objectives and rationales

Some key enzymes involved in glycolysis and the pentose phosphate bypath have been reported to be critical to the glycerol synthesis cycle in *Dunaliella*. The PFK enzyme, one of the rate-limiting enzymes, may be an important enzyme involved in regulating glycerol synthesis.

PFK catalyzes the energy producing step in the preparatory phase of glycolysis pathway (Figure 2-6). Activation of PFK leads to an upstream activation of glycolysis, which is a precondition for glycerol synthesis. Furthermore, activation of PFK enhances the carbon flux from glucose to the substrate DHAP for glycerol synthesis. Since some glucose comes from starch break-down, where starch is one important carbon pool in green cells, PFK may play major roles to trigger the glycerol synthesis and regulate glycerol synthesis from starch. However, the exact functions of this enzyme involving in glycerol synthesis have not been confirmed. Study of PFK will give more information on the regulation of glycerol synthesis in *Dunaliella*.

As the whole genomic information of *D. tertiolecta* is not available, a *PFK* cDNA sequence was cloned using the Rapid Amplification of

cDNA Ends (RACE) technology with the aim to investigate its function in glycerol production by *D. tertiolecta*. The gene was named as *DtPFK* (NCBI GenBank accession number: KJ930517) and its expression level in response to osmotic shock was evaluated. Besides, a *DtPFK* gene knock-down strain of *D. tertiolecta* (*D. tertiolecta* PR3) was generated by RNA interference (RNAi) and a glass beads mediated transformation method. Analysis of glycerol production by *D. tertiolecta* PR3 suggested that *DtPFK* may not be involved in the osmo-regulation in *D. tertiolecta* and starch break-down could have limited contribution to glycerol synthesis in the osmo-adaptation process.

4.2 Materials and Methods

4.2.1 The algal and bacterial strains and culture conditions

The alga *D. tertiolecta* and growth condition for liquid culture in flasks were the same as described in Chapter 3. *D. tertiolecta* wild-type (WT) and transformed *D. tertiolecta* colonies were grown on ATCC-1174 DA solid medium (1.2% agar) containing different NaCl concentrations, as well as in 96-well plates and 6-well plates containing ATCC-1174 DA liquid medium with different NaCl concentrations, and incubated under the same light condition at 25 °C.

The *Escherichia coli* bacterial strain used for DNA cloning was XL1-Blue, which was grown in liquid LB medium with 100 rpm shaking or on LB agar (1.5%) plates at 37 °C. The *Agrobacterium tumefaciens* strain

used for *D. tertiolecta* transformation was GV3101. Agrobacteria was grown in liquid LB medium or LB agar (1.5%) plates at 28 °C.

4.2.2 Osmotic shock treatment

D. tertiolecta wild-type (WT) cells or *DtPFK*-RNAi transformants were treated with osmotic shock following the process described in chapter 3. Samples for glycerol determination and RNA extraction were collected at certain time points. Measurement of glycerol content was performed immediately after collection. Samples for RNA extraction were frozen in liquid nitrogen before proceeding to extract total RNA or keeping at -80 °C for storage.

4.2.3 RNA extraction and total cDNA synthesis

Total RNA from *D. tertiolecta* cells was isolated using the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality and concentration was measured by NanoDrop (NanoDrop 2000 UV-Vis Spectrophotometer, Thermo Scientific) after extraction. *D. tertiolecta* total cDNA was synthesized by reverse transcription with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) following the manufacturer's instructions and synthesized cDNA concentration and quality was measured by NanoDrop.

4.2.4 PCR and molecular cloning

PfuUltra system was used in gene cloning and sequencing. DreamTaq Green system was used in colony PCR and genotyping PCR. Polymerase Chain Reaction (PCR) mixes for PfuUltra (Agilent) system and DreamTaq Green (Thermo Scientific) system are listed in Table 4-1 and Table 4-2 respectively. The thermal cycling program is listed in Table 4-3.

PCR products or plasmid vectors were digested, if necessary, with restriction enzymes (New England Biolabs) at the conditions suggested by the manufacturer. The digested products were purified using agarose gel electrophoresis and the QIAquick Gel Extraction Kit (QIAGEN), or using the QIAquick PCR Purification Kit (QIAGEN), depending on the size of the desired fragment. Ligation reaction was set up using the T4 DNA Ligase (New England BioLabs). Ligation products were transformed into *E. coli* competent cells using a heat shock transformation protocol (Inoue, Nojima et al. 1990). Transformed *E. coli* was selected on LB agar plates containing antibiotics. Single colonies were picked and colony PCR was performed for verification of the clones using colony PCR primers according to the constructs.

For cloning of the gene, the pGEM®-T Easy Vector System (Promega) was used according to the instruction manual.

4.2.5 Plasmid extraction and sequencing

Positive clones were selected for plasmid extraction and sequencing. Plasmids were extracted and purified using GeneJET Plasmid Miniprep Kit (Thermo Scientific) by following its manual instructions. Quality and concentration of extracted plasmid were measured by NanoDrop. Plasmids were sequenced by 1st BASE (www.base-asia.com). The outcomes were subjected to BLAST alignment at the NCBI website (http://www.ncbi.nlm.nih.gov/) and analyzed by software GENtle (Manske 2006). Correct constructs were stored at -20 °C for further usage.

Table 4-1: PCR reaction mix for PfuUltra (Agilent)

Components	Stock conc.	Final conc.	Volume/Reaction
PfuUltra II buffer	10 ×	1 ×	5 µl
dNTP	10 mM	200 µM	1 µl
Forward primer	10 µM	0.5 µM	2.5 µl
Reverse primer	10 µM	0.5 µM	2.5 µl
Template		2 ng/µl	100 ng
PfuUltra polymerase	2.5 U/µl	0.05 U/µl	1 µl
dd-autoclaved-water			Top up to 50 µl

Table 4-2: PCR reaction mix for DreamTaq (Thermo Scientific)

Components	Stock conc.	Final conc.	Volume/Reaction
DreamTaq Green buffer	10 ×	1 ×	2.5 µl
dNTP	10 mM	200 µM	0.5 µl
Forward primer	10 µM	0.5 µM	1.25 µl

Reverse primer	10 µM	0.5 µM	1.25 µl
Template		2 ng/µl	100 ng
PfuUltra polymerase	5 U/µl	0.025 U/µl	0.125 µl
dd-autoclaved-water			Top up to 25 µl

Table 4-3: PCR thermal cycling program

Step	Program
1	95 °C, 5 min
2	95 °C, 30 s
3	Tm* °C, 30 s
4	72 °C, X** min
5	Cycle from Step 2 to Step 4, 29 (or 34) times
6	72 °C, 10 min
7	4 °C, 10 min

* Tm is determined by the primers in the reaction.
** X is the extension time, which is 1 min per kb of the PCR fragment for DreamTaq or 1.5 min per kb for PfuUltra.

4.2.6 Cloning of *DtPFK* gene fragments in *D. tertiolecta* by RACE

Design of RACE primers for *D. tertiolecta PFK* gene (*DtPFK*) was based on the conserved regions of PFK homologues from *D. salina* (NCBI GenBank: ADE19117.1), *Chlamydomonas reinhardtii* (NCBI Reference Sequence: XP_001696305.1), *Volvox carteri* (NCBI Reference Sequence: XP_002946149.1), yeast *Saccharomyces cerevisiae* (NCBI Reference Sequence: NP_011756.1) and *Arabidopsis thaliana* (NCBI Reference Sequence: NP_194651.1), and

the primer sequences were minimized according to the *D. tertiolecta* codon bias (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3047&aa=1&style=GCG) or the PFK sequence of *D. salina*.

For cloning of *DtPFK* fragments in *D. tertiolecta*, the degenerate forward primers DtPFK-dgF1 (5'-TGCCCAAGTCGATAGACAACGAC-3') and DtPFK-dgF2 (5'-GCCTGGTGAAGCTCATGGG-3') were designed from the conserved *D. salina* PFK peptide sequences VPKSIDND and LVKLMG, while the degenerate reverse primer DtPFK-dgR1 (5'- TGGCCCAGCACCTTGCAGTA-3') and DtPFK-dgR2 (5'-TTCTGCCCTGCACCCTCCG-3') were designed from the highly conserved peptide sequences YCKVLGQ and EGAGQN. PFK gene related cDNA fragments were sequenced after amplifying by PCR.

4.2.7 5’- and 3’-RACE of *DtPFK* in *D. tertiolecta*

The 5’-RACE and 3’-RACE were performed to clone the 5’- and 3’-ends of *DtPFK* gene respectively using SMART™ RACEs cDNA Amplification Kit (Clontech, Mountain View, CA, USA). *DtPFK* 5’-RACE product was generated using UPM or NUP from the kit as forward primer and a *DtPFK* gene-specific reverse primer DtPFK-gsR1 (5'-CAATATCTGCCAGGATGGGGTT-3') or DtPFK-gsR2 (5'-CCCTTTGAAGCACCCTTCACAA-3'). *DtPFK* 3’-RACE product was generated using a gene-specific forward primer DtPFK-gsF1 (5'-CTCCATGGCATCAGGTGTTGTG-3') or DtPFK-gsF2 (5'-ATGTGTGCCTCATCCCTGAGATT-3') and reverse primer UPM or

NUP. The RACE-PCR products were cloned into pGEM®-T Easy Vector System (Promega) for sequencing.

4.2.8 Sequence analysis

RACE 5'- and 3'-overlapping sequences were assembled using EMBOSS merger program to obtain full-length sequence. The assembled sequence was named *DtPFK* (NCBI GenBank accession number: KJ930517) and analyzed for open reading frame (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequence was compared with previously characterized protein sequences by Blast (http://www.ncbi.nlm.nih.gov/BLAST/).

4.2.9 Construction of *DtPFK*-RNAi plasmid

The pGreen-0229 binary vector HY104 (Yu, Ito et al. 2004) was modified and used as *D. tertiolecta* transformation vectors. An 851-bp adenine methyltransferase (*AMT*) gene (NCBI GenBank: X06618.1) promoter from *Chlorella virus* was amplified and added into pGreen-0229 in front of the target gene to drive its expression in *D. tertiolecta*, using the PCR primer pair AMT-F-KpnI (5'-aaaGGTACCATCAGTAATGTGTTAATTGC-3'), which introduces a cutting site for the restriction enzyme *KpnI*, and AMT-R-XhoI (5'-ccgCTCGAGTTAAATAATATATAGTGTATTTTAG-3') with the *XhoI* site. A 526-bp bleomycin resistance gene (*ble*) derived from *Streptoalloteichus hindustanus* was amplified and inserted to the pGreen-0229 backbone as a dominant selectable marker to confer

zeocin-resistance, using the primers ble-F-XhoI ((5′-AAACTCGAGATGGCCAAGCTGACCAGC-3′), introducing an *XhoI* site, and ble-R2-ClaI (5′-CACATCGATTTAGTCCTGCTCCTCGGC-3′) introducing a *ClaI* site.

To generate the *DtPFK* knock-down constructs, a DNA fragment containing 432-bp of *DtPFK* coding DNA sequence (CDS) (bp 1047-1478) was selected to construct *DtPFK*-RNAi, which expressed self-complementary or hairpin RNA-containing sequences homologous to the target *DtPFK* gene driven by the *AMT* promoter. Gene sense fragment was amplified from the total cDNA of *D. tertiolecta* by PCR (Table 4-1 and Table 4-3), with primer pair DtPFK-SF-PstI (5'-AACTGCAGGCTTTGGCTTTGAGACAGCA-3') introducing a *PstI* site, and DtPFK-SR-BamHI (5'-CGGGATCCGGATGGCACGGATCATGTAA-3'), which introduces a *BamHI* site. The anti-sense sequence fragment was amplified by PCR using the primer pair DtPFK-AsF-ClaI (5'-CCATCGATGGATGGCACGGATCATGTAA-3') which introduces a *ClaI* site and DtPFK-AsR-EcoRI (5'-GGAATTCGCTTTGGCTTTGAGACAGCA-3') introducing an *EcoRI* site. The amplified gene-specific anti-sense and sense fragments were then inserted into the modified pGreen-0229 vector before and after the β-glucuronidase encoding gene (*GUS*) (Figure 4-4).

4.2.10 *D. tertiolecta* transformation using the *Agrobacterium* mediated transformation method

Agrobacteria competent cells were prepared before transformation. The GV3101 *Agrobacterium* culture with a density around 1.0 at OD_{600} was ice incubated for 20 min and spun down at 6000 rpm for 10 min at 4 °C. Supernatant was decanted and pellet was rinsed and resuspended in 1 mL ice water and mixed with an equal volume of glycerol. The suspension was aliquot 100 µl into each 1.5 mL Eppendorf tube and immediately frozen in liquid nitrogen and kept at -80 °C for long-term storage.

Constructed plasmids were transformed into *D. tertiolecta* cells using an *Agrobacterium* mediated *Dunaliella* transformation method as described before (Kumar, Misquitta et al. 2004; Fang, Lin et al. 2012) with slight modifications. The generated *DtPFK*-RNAi construct was transformed into *Agrobacterium tumefaciens* GV3101 via electroporation (2.5 kV). The transformed agrobacteria were selected by LB plates containing 50 mg mL^{-1} rifanpycin, 50 mg mL^{-1} gentamycin, 50 mg mL^{-1} kanamycin and 20 mg mL^{-1} tetramycin. Single colonies grown within 48 hours were picked and colony PCR was performed (primer set: AMT-seq-F 5'-GGTTGTTGCGAGAAATTTTG-3' and GUS102-R 5'-AACGGTTTGTGGTTAATCAGG-3').

To transform the plasmid of interest into *D. tertiolecta*, pure culture of positively transformed agrobacteria were co-cultured with logarithmic phase (5-days-old) *D. tertiolecta* liquid culture in 0.05 M NaCl 1174 DA

medium with 100 µM acetosyringone for 2 days. The transformed *D. tertiolecta* were plated onto 0.5 M NaCl 1174 DA medium agar plates containing 20-25 µg mL^{-1} zeocin (Zeocin™, Invitrogen) as the selection marker. Colonies that appeared within 2 weeks were subcultured in 96-well plates containing liquid selective medium (0.5 M NaCl ATCC-1174 DA medium containing 20 µg mL^{-1} zeocin).

4.2.11 Selection of *DtPFK* knock-down transformants by genotyping PCR

Individual *D. tertiolecta* colonies (transformants) were subsequently transferred from 96-well plates into 6-well plates containing 2 M NaCl ATCC-1174 DA medium for genotyping PCR to confirm the existence of gene integration. The purpose of increasing salinity of the culture medium was to kill the agrobacteria that survived after transformation.

For genotyping, 5 ml of *D. tertiolecta* transformants were spun down at 5000 × g for 10 minutes, 4 °C. Genomic DNA for each transformants as well as wild-type was extracted based on a simple DNA purification method (Weeks, Beerman et al. 1986). The concentration and quality of DNA were determined by NanoDrop. Genotyping PCR was performed using the genomic DNA samples as templates and primers ble-gen-F (5'-GGAGCGGTCGAGTTCTGG-3') and ble-gen-R (5'-CTCGCCGATCTCGGTCAT-3').

4.2.12 Gene expression analysis by QRT-PCR

The relative gene expression was analyzed by quantitative real-time (QRT-) PCR with the ABI PRISM® 7500 Real-Time PCR System (Applied Biosystems) using the Maxima® SYBR Green/ROX qPCR Master Mix 2× (Fermentas) according to the manufacturer's instructions. *D. tertiolecta β-tubulin* gene (*DtTUB*, sequence amplified by laboratory colleagues) was used as the endogenous control. The primers targeted against sequences of the *DtPFK* gene are DtPFK-rtF (5'-CTCCATGGCATCAGGTGTTGTG-3') and DtPFK-rtR (5'-CAATATCTGCCAGGATGGGGTT-3'). The primers for *DtTUB* gene are DtTUB-rtF (5'-CAGATGTGGGATGCCAAGAACAT-3') and DtTUB-rtR (5'-GTTCAGCATCTGCTCATCCACCT-3'). Each pair of gene-specific primers used in the QRT-PCR was checked by the specificity of the melting curve.

All reactions were carried out in parallel triplicates and the experiments were repeated 3 times. Quantification of RNA was based on Ct (threshold cycle) values. The Ct values of *DtPFK* were normalized using the Ct value corresponding to *DtTUB*. The efficiency of each QRT-PCR was also calculated. Data analysis was executed using comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001).

4.3 Results

4.3.1 Identification and characterization of *DtPFK* in *D. tertiolecta*

A *PFK* cDNA homologue was isolated from *D. tertiolecta* using RACE and named *DtPFK*. Sequencing of the *DtPFK* gene showed its CDS consists of 1558-bp and encodes a predicted protein containing 340 amino acids. A BLAST search against the NCBI database showed that the deduced amino acid sequence shared a 99% similarity to its homologous protein in *D. salina* (DsPFK) and approximately 80% similarity to that in *C. reinhardtii* or *Volvox*. The protein alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) also suggests that DtPFK is highly conserved and has a close relationship with PFK proteins in other green microalgae (Figure 4-1). The PFK superfamily protein specific hits (including an active site, a fructose-1, 6-bisphosphate-binding site, an ADP/pyrophosphate-binding site, an allosteric effector site and a dimerization interface) are located between position 27 and position 266.

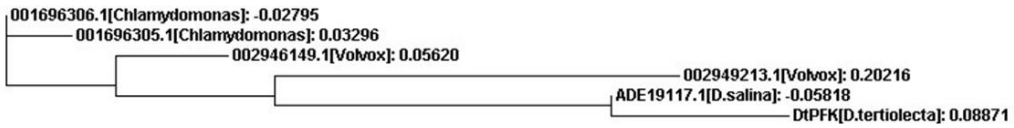

Figure 4-1 Protein alignment of the deduced amino acid sequence of *DtPFK* NCBI referencing numbers for PFKs in other organisms are shown. Alignment was generated using the website (http://www.ebi.ac.uk/Tools/msa/clustalo/).

4.3.2 Expression of *DtPFK* in *D. tertiolecta* in response to hyper- or hypo-osmotic shock

Expression of *DtPFK* gene at the transcriptional level upon hyper- or hypo-osmotic shock is shown in Figure 4-2. The mRNA level of *DtPFK* was increased approximately 9 folds upon hyper-osmotic shock transiently within the first two hours (Figure 4-2 A), but suppressed by hypo-osmotic shock (Figure 4-2 B). As glycerol synthesis was also up-regulated upon hyper-osmotic shock and suppressed upon hypo-osmotic shock according to the physiological study in Chapter 3, it is suggested that *DtPFK* might be involved in the osmo-response of *D. tertiolecta* and the osmo-regulation of glycerol production.

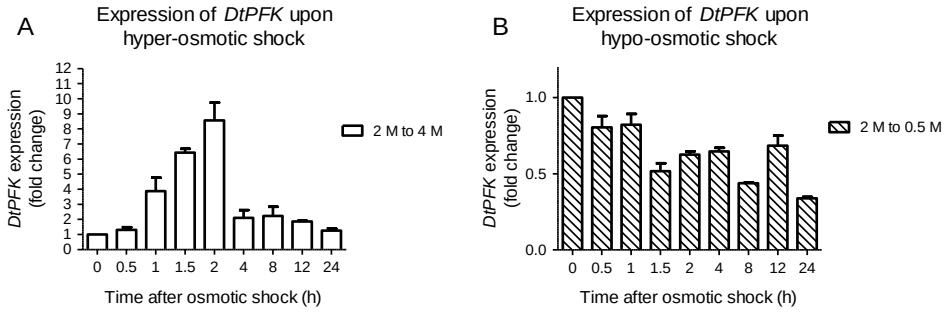

Figure 4-2 Fold change of *DtPFK* mRNA level upon osmotic shock
Values shown are the means of three independent experimental sets. *DtPFK* expression level at time 0 (before treatment) was normalized as 1 for each set of experiment. Fold change (Average of three sets of experiment) was calculated based on the isotonic control group (2 M to 2 M) taken at the same time points. *DtTUB* was used as the endogenous reference. A: Fold change of *DtPFK* gene expression upon hyper-osmotic shock (2 M to 4 M); B: Fold change of *DtPFK* gene expression upon hypo-osmotic shock (2 M to 0.5 M).

4.3.3 *DtPFK* expression under various osmotic stresses

As described in 3.2.3 of Chapter 3, enhancement of *D. tertiolecta* glycerol production was observed upon hyper-osmotic shock for the

whole cell growth period, and glycerol production is higher at higher salinities when *D. tertiolecta* were subjected to various long-term osmotic stresses.

With the attempt to learn whether *DtPFK* was involved in the long-term regulation of glycerol production, its gene expression level under long-term osmotic stress conditions was investigated. *D. tertiolecta* cells growing in ATCC-1174 DA medium containing 2 M NaCl were harvested at the logarithmic phase and treated with osmotic shock to 0.05 M, 1 M, 2 M, 3 M or 4 M NaCl. *DtPFK* mRNA level was measured after 5 days' prolonged osmotic stress exposure, as shown in Figure 4-3. It revealed that the steady state expression level of this gene was positively correlated with salinity, such that higher gene expression was observed at higher salt condition. This finding is consistent with the result that higher glycerol was produced under higher salt stress, which suggests that *DtPFK* may be related to glycerol synthesis in *D. tertiolecta* in response to osmotic shock.

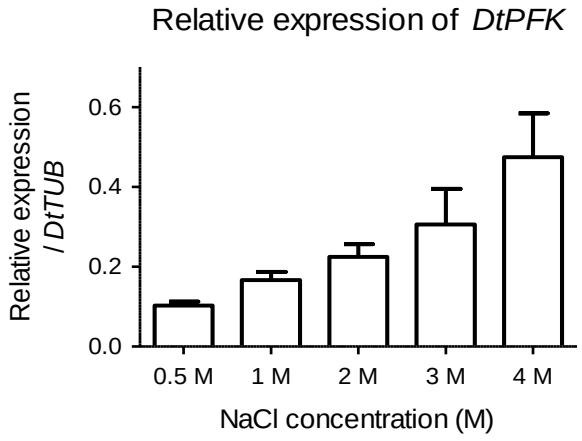

Figure 4-3 *DtPFK* expression level of *D. tertiolecta* grown in different osmotic stress conditions

DtTUB was used as the endogenous reference. Data shown were the average of triplicate experiments conducted in parallel. *D. tertiolecta* cells at logarithmic growth phase growing in 2 M NaCl ATCC-1174 DA medium were harvested and resuspended into ATCC-1174 DA medium containing 0.05 M, 0.5 M, 1 M, 2 M, 3 M or 4 M NaCl accordingly for different osmotic shock treatments. Relative gene expression level was measured on the 5th day (120 h) after osmotic shock treatment.

4.3.4 Generation and selection of the *DtPFK* knock-down transformants

The *DtPFK*-RNAi plasmid was constructed as described in section 4.2.9, and the schematic diagram of the construct is presented in Figure 4-4. A 432-bp fragment of the anti-sense and sense sequence of *DtPFK* gene CDS forms the hairpin structure with the *GUS* loop.

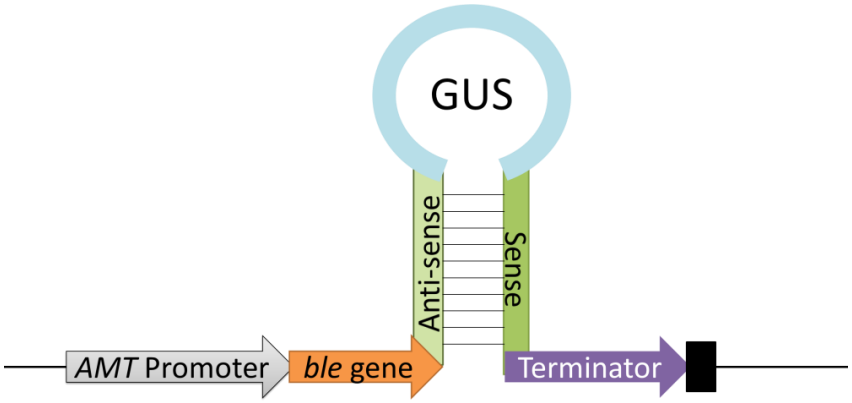

Figure 4-4 Schematic diagram of *DtPFK*-RNAi construct
Anti-sense and Sense: anti-sense sequence and sense sequence of *DtPFK* gene CDS fragment.

After transformation, 96 colonies (one 96-well plate) were picked from the zeocin-containing agar plates before the culture were enlarged in 6-well plates for DNA extraction, and then selected by genotyping PCR. Genotyping-positive colonies showed a PCR product band, which was 397-bp, as shown in Figure 4-5. It can be seen that even with the same amount of genomic DNA used as template, the PCR product amount

could be different. This might be caused by the different insertion efficiency of the target gene into genome during transformation. Genotyping-positive transformants showing abundant PCR products (similar as Lane 4 or Lane 7 in Figure 4-5) were selected (S1-S11) and subjected to further selection by QRT-PCR (Figure 4-6).

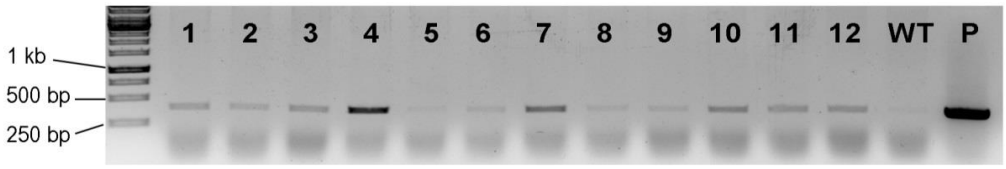

Figure 4-5 Genotyping PCR for *DtPFK* knock-down transformants selection
Lane 1-12: 12 colonies picked after transformation; WT: negative control using *D. tertiolecta* wild-type genome as template; P: positive control using *DtPFK*-RNAi plasmid as template.

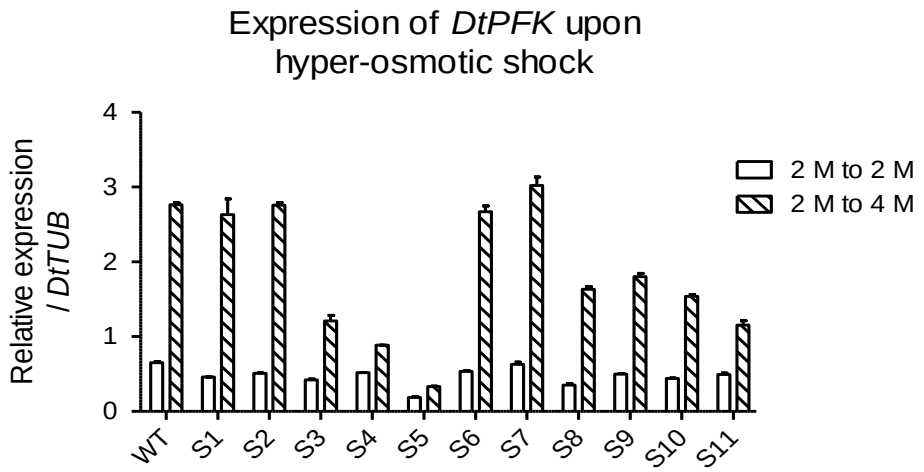

Figure 4-6 QRT-PCR results for *DtPFK* knock-down transformants selection
DtTUB was used as the endogenous reference. WT: *D. tertiolecta* wild-type; S1-S11: 11 genotyping-positive transformants selected by genotyping PCR. 2 M to 2 M (unshaded), isotonic control; 2 M to 4 M (shaded), cells were shifted from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl for treatment of hyper-osmotic shock. Samples were taken at 2 hours after treatment. Data shown are the average of technical triplicates in one set of osmotic shock treatment experiment.

Successful transformed *DtPFK* knock-down mutants were finally selected and confirmed by the expression level of *DtPFK*. Transformants (S1-S11) selected by genotyping PCR were treated with hyper-osmotic shock, in comparison with wild-type. The expression

level of *DtPFK* was determined 2 hours after treatment. Figure 4-6 presents the relative expression level of *DtPFK* in different transformed strains as well as wild-type. *DtPFK* expression level in *D. tertiolecta* wild-type could be highly up-regulated 2 hours after the hyper-osmotic shock from 2 M to 4 M NaCl (Figure 4-2 A). As can be seen, some of the transformants had similar responses to wild-type, i.e. expression of *DtPFK* could be up-regulated upon 2 M to 4 M hyper-osmotic shock, while some other transformants, especially S5, displayed no obvious up-regulation on the gene expression level. Besides, when comparing the 2 M to 2 M isotonic controls of all these strains, *DtPFK* expression level in S5 was also lower than that of wild-type. This suggests that S5 is a successful *DtPFK* knock-down strain. The S5 was then named PR3 and subjected to further study on its osmo-regulation of gene expression as well as glycerol production.

4.3.5 *DtPFK* expression in the *DtPFK* knock-down strain PR3

The effect of *DtPFK* gene knock-down was confirmed in PR3 by triplicate hyper-osmotic shock experiments. Figure 4-7 shows the average up-regulated fold on *DtPFK* expression 2 hours after hyper-osmotic shock from 2 M to 4 M NaCl. The result displayed no change in *DtPFK* expression level when PR3 cells were treated with 2 M to 4 M hyper-osmotic shock, although PR3 had similar expression level at 2 M to 2 M condition,. This result confirms that the expression of *DtPFK* in PR3 was at low level and could not be up-regulated upon hyper-

osmotic shock treatment, suggesting the successful knock-down of *DtPFK* in PR3.

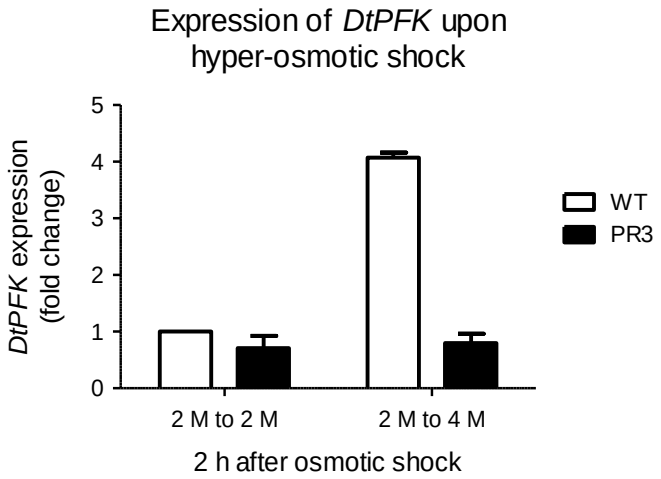

Figure 4-7 Expression of *DtPFK* in the knock-down strain PR3 could not respond to hyper-osmotic shock
DtPFK expression level in the wild-type isotonic control group (2 M to 2 M) was normalized as 1 for each set of experiment. The average fold change of three sets of experiments was shown. *DtTUB* was used as the endogenous reference. WT (unshaded): *D. tertiolecta* wild-type; PR3 (black): *DtPFK* knock-down strain PR3. 2 M to 2 M, isotonic control; 2 M to 4 M, cells treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl. Samples were taken at 2 hours after osmotic shock treatment.

4.3.6 Glycerol production by the *DtPFK* knock-down strain PR3

Glycerol production by PR3 was determined in order to investigate whether *DtPFK* is involved in the regulation of glycerol synthesis in *D. tertiolecta*. Figure 4-8 presents the glycerol production by PR3 under the hyper-osmotic shock condition in comparison with *D. tertiolecta* wild-type. Both wild-type and PR3 displayed similar pattern of glycerol production variation in response to hyper-osmotic shock.

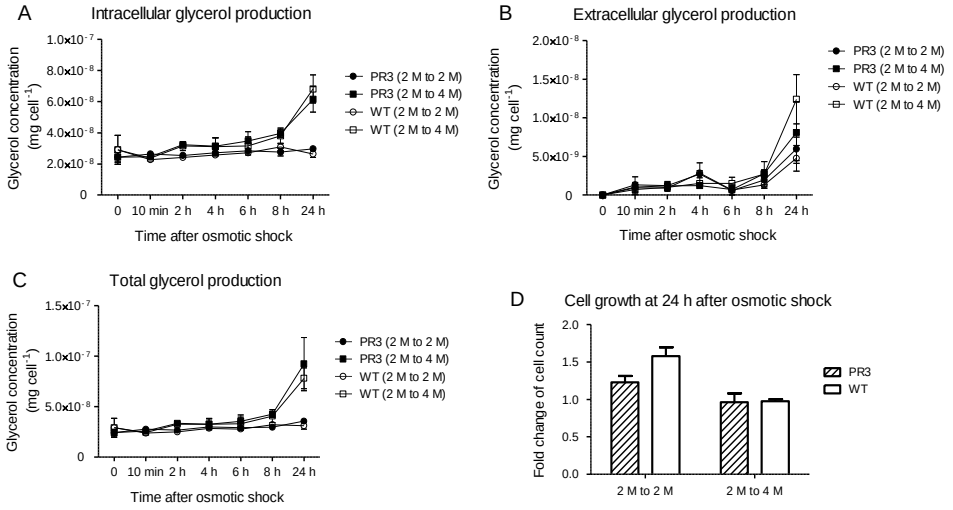

Figure 4-8 Glycerol determination of the *DtPFK* knock-down strain PR3 in response to hyper-osmotic shock
PR3 (2 M to 2 M) (black circle): isotonic control of PR3; PR3 (2 M to 4 M) (black square): PR3 treated with hyper-osmotic shock; WT (2 M to 2 M) (white circle): isotonic control of *D. tertiolecta* wild-type; WT (2 M to 4 M) (white square): *D. tertiolecta* wild-type treated with hyper-osmotic shock. A: Intracellular glycerol production (mg per cell) by PR3 and *D. tertioelecta* wild-type; B: Extracellular glycerol production; C: Total glycerol production. D: Fold change of cell count at 24 h after osmotic shock (24 h); PR3 (shaded): *DtPFK* knock-down strain PR3; WT (unshaded): *D. tertiolecta* wild-type. Values shown are the means of three independent sets of experiment.

The intracellular glycerol content was maintained at a steady level in the 2 M to 2 M isotonic control group and doubled in the 2 M to 4 M hyper-osmotic shock condition in both wild-type and PR3 cells (Figure 4-8 A). The extracellular glycerol had high producing rate under hyper-osmotic stress (Figure 4-8 B), thus the total glycerol production is higher under hyper-osmotic shock condition (Figure 4-8 C). However, there was no difference in regulation on glycerol content between PR3 and wild-type. The only difference observed between these two strains was that the cell growth might be affected by the knock-down of *DtPFK* gene: Figure 4-8 D shows the fold change of cell numbers in PR3 was lower compared to wild-type (WT) after 24 hours in 2 M NaCl ATCC-1174 DA medium. *D. tertiolecta* had inhibited cell growth in 4 M NaCl

ATCC-1174 DA medium, with a doubling time of approximately 80 hours (Chapter 3), thus cell numbers of both strains had nearly no change when salinity was changed from 2 M to 4 M. However, when transferred from 2 M to the isotonic fresh ATCC-1174 DA liquid medium, the PR3 shows a slightly slower growth rate than wild-type. This suggested that cell growth of PR3 may be inhibited due to the knock-down of *DtPFK* gene, while the glycerol production was not affected.

4.4 Discussion

Gene sequence analysis and protein alignments with homologues in other *Dunaliella* species suggest that this enzyme is highly conserved and DtPFK has close relationship with its homologous proteins in other green microalgae. This suggests that genes in *Dunaliella* are highly conserved. Being the ancestor of green plants, *Dunaliella* can be studied as a good model for the salt tolerance mechanisms and stress responses in crops and other higher plants.

PFK has been reported to be an important checkpoint enzyme in the activation of glycerol synthesis (Chitlaru and Pick 1991). A decrease in fructose 6-phosphate concentration corresponded with an increase in fructose-1, 6-bisphosphate when *D. salina* exposed to hyper-osmotic shock, which suggests that PFK is activated for glycerol synthesis from glucose derived from starch degradation (Chitlaru and Pick 1991). The transcriptional study showed that *DtPFK* mRNA levels could be transiently regulated accordingly: up-regulated after hyper-osmotic

shock while down-regulated after hypo-osmotic shock, suggesting a corresponding to glycerol production after osmotic shock. Therefore, up-regulation of *DtPFK* gene could be a pre-condition for rapid glycerol synthesis in response to extracellular osmotic change. When cells were grown at different salinities, there was also a positive relationship between the expression level of *DtPFK* and the medium salt concentration. The up-regulation of *DtPFK* by hyper-osmotic stress suggests that *DtPFK* is required for glycolysis which provides intermediates for glycerol synthesis through starch degradation.

Further evidence for the involvement of *PFK* in the regulation of glycerol synthesis is that *PFK* in budding yeast was found to be essential for its glycerol accumulation as this *PFK* gene could be induced by HOG pathway to be activated under hyper-osmotic stress (Dihazi, Kessler et al. 2004). Activation of this *PFK* gene leads to the activation of the preparatory phase of glycolysis, and this was suggested to be a precondition for glycerol accumulation.

In addition, the enhancement of *D. tertiolecta* glycerol production under hyper-osmotic stress lasted for the whole growth period. Gene expression level of *DtPFK* in *D. tertiolecta* was positively correlated with external salinity when exposed with long-term hyper-osmotic conditions (5 days) (Figure 4-3). Higher gene expression level was observed at higher salt condition. These findings reveled that *DtPFK* may not only function in the short-term response to osmotic change, but also play important roles when regulating the glycerol synthesis in

long-term adaptation. This gives more information on the regulating mechanisms and signalling pathways for glycerol synthesis in *D. tertiolecta*.

However, through the gene expression study shows a correlation between the glycerol synthesis and the expression of *DtPFK*, glycerol production by the generated gene knock-down strain PR3 did not show significant difference on glycerol production compared to wild-type upon osmotic shock. Considering the much smaller cell size of *D. tertiolecta* (Lin, Fang et al. 2013), a possible reason could be that starch storage was too low in *D. tertiolecta* as the amount is negligible compared to the carbon required for glycerol synthesis (Chapter 3). PFK is the classical checkpoint enzyme in glycolysis pathway (GIMMLER and MÖLLER 1981; Chitlaru and Pick 1991), while glycolysis is the major pathway for starch break-down (Figure 2-6),it can be suggested that starch degradation or glycolysis pathway may not be an important carbon provider for glycerol synthesis in *D. tertiolecta* under osmotic shock conditions. Starch degradation has been suggested as an important carbon source for glycerol synthesis in *D. tertiolecta* beside photosynthetically fixed carbon (Liska, Shevchenko et al. 2004; Goyal 2007; Goyal 2007). It appears that relative contributions of these two metabolic pathways were different in different species of *Dunaliella*, as starch degradation may have very limited contribution as a carbon provider for glycerol synthesis in *D. tertiolecta*. What's more, cellular starch content was found to be negligible compared to the glycerol production in response to osmotic

shock (Chapter 3), suggesting that starch break-down provided negligible amount of carbon for glycerol synthesis upon osmotic shock. Therefore, starch degradation or glycolysis pathway may not be needed for glycerol synthesis and the osmo-regulation in *D. tertiolecta*, as reported in other species of *Dunaliella* as well as the budding yeast.

Knock-down of PFK gene may lead to inhibition of glycolysis pathway, which generates ATP and NADPH used in reductive synthetic pathways in cells, thus resulting in a slight inhibition of cell growth (Figure 4-8 D). As glycerol production on a per cell basis was not affected compared to wild-type cells. It seems that the carbon used for cell growth would flow to glycerol in order to produce enough glycerol to counter-balance the osmotic stress. These findings suggest that glycolysis pathway would rather play a major role in regulating cell growth but may not be regulating glycerol synthesis in *D. tertiolecta*.

The findings demonstrated that *DtPFK* may not be crucial for the rapid regulation of glycerol synthesis in response to osmotic shock. However, another possibility could be that DtPFK functions at the protein level to regulate glycerol synthesis. Although the techniques for study at protein level in *Dunaliella* are not convenient, specific protein antibodies for DtPFK may be generated in order to identify the function of this enzyme involved in regulation of cell growth or glycerol synthesis. Further study on DtPFK enzymatic activities can also be conducted to give more information on its catalytic role in glycolysis

and to evaluate whether it would regulate glycerol production in *D. tertiolecta.*

4.5 Conclusion

Starch breakdown may not contribute to glycerol synthesis in *D. tertiolecta* as much as in other species such as *D. salina*. As the photosynthetic activity also has different response to osmotic shock in *D. tertiolecta* compared to what have been reported in both *D. salina* and *D. bardawil*, different mechanisms for glycerol synthesis and regulation might be involved in regulating the osmotic adaptation process in different species of *Dunaliella.* For a more detailed understanding of the osmo-regulatory mechanisms for *D. tertiolecta* glycerol synthesis, research was extended in next chapter to reveal other molecular mechanisms that might be involved.

CHAPTER 5 *MAPK* GENE INVOLVED IN REGULATING GLYCEROL PRODUCTION UNDER OSMOTIC STRESS IN *DUNALIELLA TERTIOLECTA*

5.1 Objectives and rationales

Glycerol production in *D. tertiolecta* can be also regulated by other mechanisms, such as a cell signaling pathway. The yeast HOG pathway is one of the well-studied signaling pathway. Transcription of GPDH homologue GPD1 is regulated by HOG pathway in yeast (Figure 2-7). Once the HOG pathway is activated by environmental cue, the activated Hog1 will migrate into the nucleus and induce the expression of GPD1, thus triggering the glycerol synthesis cycle. MAPK cascade that is similar to yeast HOG pathway may be involved in *Dunaliella* in the mechanism to regulate glycerol production and osmo-responses. It would be of great importance in uncovering the molecular basis for the osmo-regulatory mechanisms if a HOG-like pathway is found to regulate glycerol synthesis in *D. tertiolecta*.

However, no MAPK has been identified in *D. tertiolecta*; neither has the whole cascade been demonstrated in *D. tertiolecta*. As the whole genomic information of *D. tertiolecta* is not available, a *MAPK* cDNA was cloned using RACE technology and named as *DtMAPK* (NCBI GenBank accession number: KJ930518). The GPDH encoding gene

DtGPDH has been isolated from *D. tertiolecta* by a lab colleague (NCBI GenBank accession number: KJ930370). While there is no information on whether there is a MAPK regulating the expression of GPDH in *D. tertiolecta*, it is of great importance to study the correlation of the identified MAPK gene *DtMAPK* and *DtGPDH*.

In addition to the gene isolation work, expression levels of *DtMAPK* and *DtGPDH* in response to osmotic shock were evaluated. A *DtMAPK* knock-down strain of *D. tertiolecta* (*D. tertiolecta* MR2) was generated with *DtMAPK*-RNAi construct and glass beads mediated transformation method. Analysis of the gene expression of *DtGPDH* in the MR2 suggested that *DtGPDH* could be regulated by *DtMAPK* at the transcription level. Determination of the glycerol production in response to osmotic shock in *D. tertiolecta* MR2 further confirmed that *DtMAPK* may be involved in the regulation of glycerol synthesis. All these findings demonstrated that *DtMAPK* can regulate the glycerol synthesis in *D. tertiolecta* by regulating the expression of *DtGPDH* gene.

5.2 Materials and Methods

5.2.1 The algal and bacterial strains and culture conditions

Organism strains and culture conditions were the same as described in chapter 3 and chapter 4.

5.2.2 Cloning of *DtMAPK* gene fragments in *D. tertiolecta*

RACE primers for *D. tertiolecta MAPK* gene (*DtMAPK*) were designed from the conserved regions of MAPK from *D. salina* (NCBI GenBank: ABN03944.1), *Chlamydomonas reinhardtii* (NCBI Reference Sequence: XP_001700291.1), *Volvox carteri* (NCBI Reference Sequence: XP_002955338.1), *Arabidopsis thaliana* (GenBank: ABR46165.1) and the budding yeast *Saccharomyces cerevisiae* (NCBI Reference Sequence: NP_013214.1). All primer sequences were minimized based on the *D. tertiolecta* codon bias from the website (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3047&aa=1&style=GCG) or the sequence of MAPK from *D. salina*.

The forward primers DtMAPK-dgF1 (5'-AGGAGCATACGGTGTGGTTTG-3') and DtMAPK-dgF2 (5'-AAAGTGGCCATCAAGAAAAT-3') were designed from the conserved peptide sequences GAYGVVC and KVAIKK. The reverse primers DtMAPK-dgR1 (5'-TAGCTCAGGGGCTCTGTACCA-3') and DtMAPK-dgR2 (5'-TAGTCCTTGCCGGGGAACA-3') were designed from the conserved peptide sequence WYRAPEL and FPGKDY. *DtMAPK* gene related cDNA fragments were amplified by PCR and then sequenced.

5.2.3 5’- and 3’-RACE of *DtMAPK* gene in *D. tertiolecta*

The 5’- and 3’- ends of *D. tertiolecta MAPK* gene (*DtMAPK*) were cloned using the SMARTer™ RACE cDNA Amplification Kit (Clontech).

DtMAPK 5'-RACE product was generated using 10×Universal Primer A Mix (UPM) or Nested Universal Primer A (NUP) provided with the kit as a forward primer and MAPK gene-specific primers DtMAPK-gsR1 (5'-ATCGCGGTGCAGGATGGCAG-3') or DtMAPK-gsR2 (5'-CCACACCGTATGCTCCTTTGCCA-3') as a reverse primer. *DtMAPK* 3'-RACE product was generated using a gene-specific forward primer DtMAPK-gsF1 (5'-TGCCAAACGCACACTGCGTG-3') or DtMAPK-gsF2 (5'-CGCCTGATGTGGTGCGAGGC-3') and UPM or NUP as a reverse primer. The RACE-PCR products were cloned into pGEM®-T Easy Vector System (Promega) for sequencing.

5.2.4 Sequence analysis

RACE 5'- and 3'-overlapping sequences were assembled using EMBOSS merger program to obtain full-length sequence. The assembled sequence was named *DtMAPK* (NCBI GenBank accession number: KJ930518) and analyzed for open reading frame (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequence was compared with previously characterized protein sequences by Blast (http://www.ncbi.nlm.nih.gov/BLAST/).

5.2.5 Construction of *DtMAPK*-RNAi plasmid

To generate the *DtMAPK* knock-down construct, a DNA fragment containing 334-bp of *DtMAPK* gene CDS (bp 75-408) was selected to construct *DtMAPK*-RNAi, which expressed self-complementary or hairpin RNA-containing sequences homologous to the target *DtMAPK*

gene driven by the *AMT* promoter. Sense and anti-sense gene sequences of these fragments were amplified from the total cDNA of *D. tertiolecta* by PCR. Primer pair for the sense fragment amplification were the forward primer DtMAPK-SF-PstI (5'-AACTGCAGAGTCCATCAACTATGAGGCCG-3'), which introduces a *PstI* site, and the reverse primer DtMAPK-SR-BamHI (5'-CGGGATCCTCGTACACCACATACAGGTCC-3') introducing a *BamHI* site. Primers for the amplification of the anti-sense fragment were DtMAPK-AsF-ClaI (5'-CCATCGATTCGTACACCACATACAGGTCC-3'), which introduces a *ClaI* site, and DtMAPK-AsR-EcoRI (5'-CGGAATTCAGTCCATCAACTATGAGGCCG-3'), which introduces an *EcoRI* site. The amplified gene-specific anti-sense and sense fragments were then inserted into the modified pGreen-0229 vector before and after the *GUS* gene fragment. The plasmid also incorporated an *AMT* promoter and *ble* gene selection marker.

5.2.6 *D. tertiolecta* transformation using the *Agrobacterium* mediated transformation method

Agrobacteria competent cells were prepared following the procedures described in Chapter 4. The constructed plasmid for *DtMAPK* knock-down was then transformed into *D. tertiolecta* cells using the *Agrobacterium* mediated *Dunaliella* transformation method as described in the previous chapter (4.2.10 in Chapter 4).

5.2.7 Selection of *DtMAPK* knock-down transformants

Transformed *D. tertiolecta* colonies were picked and genotyping PCR was performed to confirm the existence of the gene integration following the method in Chapter 4. The positive strains were further analyzed for gene expression level and glycerol production after osmotic shock treatment following the process below.

5.2.8 Osmotic shock treatment

D. tertiolecta wild-type (WT) cells or *DtMAPK*-RNAi transformants were treated with osmotic shock following the process described in previous chapters 3 and 4. Samples were taken at certain time points and subjected to measurement of glycerol content. For RNA extraction, samples were frozen in liquid nitrogen before extraction immediately or kept at -80 °C for storage.

5.2.9 Cell size measurement

Both *D. tertiolecta* wild-type and the *DtMAPK* gene knock-down strain were treated with hyper-osmotic shock following the above stated procedures. Cells at 24 hours after treatment were photographed at 1000 times magnification under a light microscope (Olympus) and cell biovolume was measured using the equation and geometric model described in Chapter 3.

5.2.10 Gene expression analysis by QRT-PCR

Total cDNA was synthesized using the extracted RNA, following the procedures described in Chapter 4. The relative gene expressions of *DtMAPK* and a key enzyme encoding gene for glycerol synthesis *DtGPDH* (sequence amplified by a laboratory colleague, NCBI GenBank accession number: KJ930370) in *D. tertiolecta* were then analyzed by QRT-PCR. The was forward primer for determination of *DtMAPK* gene expression was DtMAPK-rtF (5'-AGCCCCTGAGCTACTGCTCTCAT-3') and the reverse primer was DtMAPK-rtR (5'-GTTCAGCTGGTGCACGTAGTCCT-3'). The primer pair for *DtGPDH* gene expression was DtGPDH-rtF (5'-ATTAACCTGCTTGCGGATGT-3') and DtGPDH-rtR (5'-CATAGCTGCTGGCAATCAAA-3'). The QRT-PCR conditions and the calculating methods were the same as described in Chapter 4, with *DtTUB* as the endogenous control.

5.3 Results

5.3.1 Identification and characterization of the *DtMAPK* cDNA in *D. tertiolecta*

Because the whole genome sequence of *D. tertiolecta* is not available, RACE was employed as a method to isolate the *MAPK* homologue in *D. tertiolecta*, which was named *DtMAPK*. The entire coding region of *DtMAPK* is 1471-bp in length, and encodes a deduced protein containing 472 amino acids. A BLAST search against the NCBI

database revealed that the amino acid sequence is 98% and 81% similarly to two MAPK proteins reported in *D. salina* (DsMPK), and 75% similarly to MAPK of *C. reinhardtii* (CrMAPK). The alignment with MAPK proteins in some other green microalgae suggested that DtMAPK protein has a close relationship with its homologous proteins DsMPK and CrMAPK (Figure 5-1). A conserved MAPK family protein catalytic domain (including an active site, an ATP-binding site, a substrate-binding site, an activation loop and a KIM docking site) of the Serine/Threonine Kinases, TEY Mitogen-Activated Protein Kinases from plants was predicted to be located from position 43 to position 241.

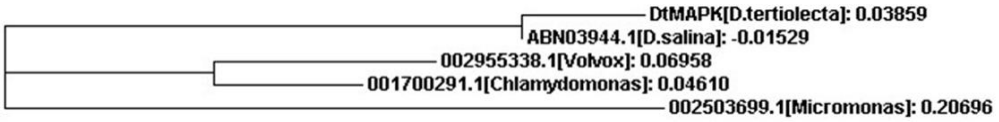

Figure 5-1 Protein alignment of the deduced amino acid sequence of *DtMAPK* NCBI reference numbers for MAPK in other organisms were shown. Sequence alignment was generated with the website (http://www.ebi.ac.uk/Tools/msa/clustalo/).

5.3.2 Expression of *DtGPDH* and *DtMAPK* under various osmotic stress conditions

GPDH catalyze the rate-limiting step for glycerol synthesis. The GPDH encoding gene *DtGPDH* has been isolated from *D. tertiolecta* by a laboratory colleague (NCBI GenBank accession number: KJ930370). To investigate whether *DtMAPK* is involved in the regulation of glycerol production, expression levels of both *DtGPDH* and *DtMAPK* were evaluated in *D. tertiolecta* under various osmotic stress conditions (Figure 5-2). The data shows that the steady state expression of both genes was positively correlated to the external salinity, such that higher

gene expression was observed at higher salt stress condition. Since higher glycerol production has been observed in higher salinity, as described in Chapter 3, the increasing expression of *DtGPDH* with increasing salinity suggested that *DtMAPK* may be related to the expression of *DtGPDH* as well as the regulation of glycerol synthesis in *D. tertiolecta* in response to osmotic shock.

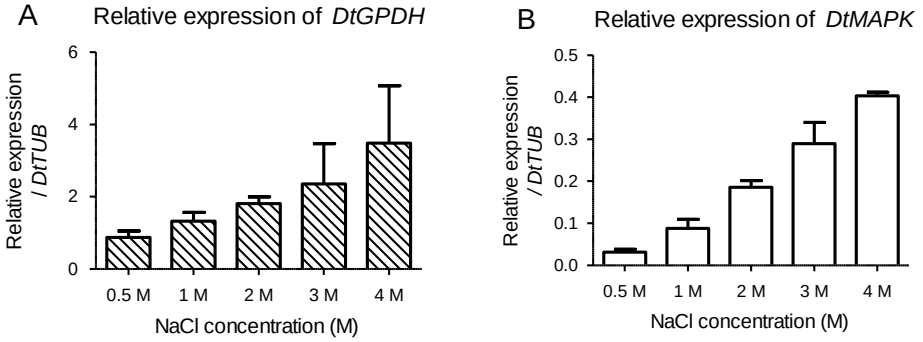

Figure 5-2 Expression level of *DtGPDH* (A) and *DtMAPK* (B) under various osmotic stress conditions
DtTUB was used as the endogenous reference. Data shown was the average of triplicate experiments conducted in parallel. *D. tertiolecta* cells at logarithmic growth phase growing in 2 M NaCl ATCC-1174 DA medium were harvested and resuspended into ATCC-1174 DA medium containing 0.05 M (hypo-osmotic shock), 0.5 M (hypo-osmotic shock), 1 M (hypo-osmotic shock), 2 M (isotonic control), 3 M (hyper-osmotic shock) or 4 M (hyper-osmotic shock) NaCl accordingly for different osmotic shock treatments. Samples were collected for RNA extraction on the 5^{th} day (120 h) after osmotic shock.

5.3.3 Expression of *DtMAPK* in response to osmotic shock

Glycerol production by *D. tertiolecta* can respond to osmotic shock rapidly. The expression kinetics of *DtMAPK* in response to osmotic shock was further investigated. Figure 5-3 shows the fold change of *DtMAPK* expression level in response to a 2 M to 4 M hyper-osmotic shock and a 2 M to 0.5 M hypo-osmotic shock. As can be seen, expression of *DtMAPK* responded to osmotic shock within 0.5 h.

DtMAPK was transiently up-regulated by approximately 5 folds within 2 h upon hyper-osmotic shock (Figure 5-3 A) while significantly suppressed upon hypo-osmotic shock within 0.5 h (Figure 5-3 B). As glycerol synthesis was also up-regulated upon hyper-osmotic shock and suppressed upon hypo-osmotic shock as shown in Chapter 3, it could be suggested that *DtMAPK* might be involved in the osmo-response and the regulation of glycerol production by *D. tertiolecta.*

However, the increased expression of *DtMAPK* under hyper-osmotic shock condition was found to return to the base level 8 hours after hyper-osmotic shock (Figure 5-3 A), while the glycerol concentration was maintained at high levels under hyper-osmotic stress. Thus *DtMAPK* may be only responsible for the rapid response and emergency osmo-regulation of glycerol synthesis in *D. tertiolecta*. Once up-regulated, the production of glycerol remained at the high level as shown in Chapter 3.

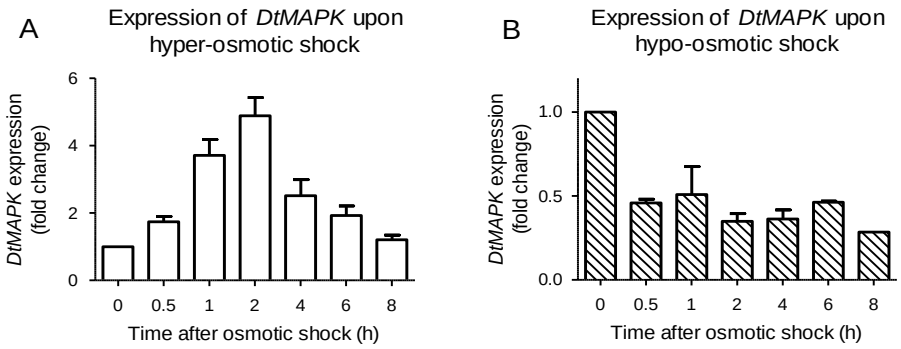

Figure 5-3 Fold change of *DtMAPK* expression level in reponse to osmotic shock

DtMAPK expression level at time 0 (before osmotic shock) was normalized as 1 for each set of experiment. Fold change (Average of three independent sets of experiment) was shown based on the isotonic control group (2 M to 2 M) at the same time points taken. *DtTUB* was used as the endogenous reference. A: Fold change of *DtMAPK* gene expression upon hyper-osmotic shock (2 M to 4

M); B: Fold change of *DtMAPK* gene expression upon hypo-osmotic shock (2 M to 0.5 M).

5.3.4 Expression of *DtGPDH* in response to hyper-osmotic shock

It has been suggested that expression of GPDH in yeast is regulated by the Hog1 protein, thus the glycerol synthesis was triggered by activation of Hog1. With the attempt to explore the correlation between *DtMAPK* and *DtGPDH* in *D. tertiolecta*, the expression level of *DtGPDH* was determined. Figure 5-4 shows the fold change of *DtGPDH* gene expression of *D. tertiolecta* in response to hyper-osmotic shock from 2 M NaCl ATCC-1174 DA medium to 4 M NaCl medium. It can be seen that *DtGPDH* was up-regulated by hyper-osmotic stress within 1 h. The expression level of *DtGPDH* was increased by 10-15 times during the 4-8 hours after hyper-osmotic shock. The increased *DtGPDH* expression could be responsible for the increased glycerol production under hyper-osmotic stress. In addition, when comparing the expression of *DtGPDH* in response to hyper-osmotic shock with that of *DtMAPK* (Figure 5-3 A), it can be seen that both genes displayed similar transient up-regulation pattern. However, the expression level of *DtMAPK* was increased 1-4 hours after osmotic shock, while the increase of *DtGPDH* expression happened during the 4-8 hours after osmotic shock. There might be a delay in the response of *DtGPDH* gene expression compared to that of *DtMAPK* if indeed the expression of *DtGPDH* gene was regulated by *DtMAPK*. The expression of *DtMAPK* was transiently up-regulated upon hyper-osmotic shock

(Figure 5-3 A). A possible reason could be that only a finite amount of DtGPDH enzyme is required to increase intracellular glycerol to the new level, which is sufficient to counteract external osmotic pressure.

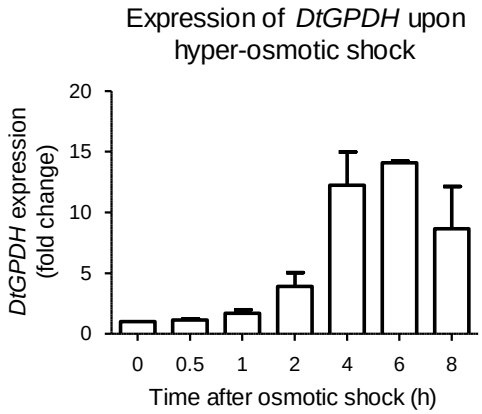

Figure 5-4 Fold change of *DtGPDH* expression level in response to hyper-osmotic shock
Logarithmic phase of *D. tertiolecta* cells were treated with hyper-osmotic shock from 2 M NaCl ATCC-1174 DA medium to 4 M NaCl medium. *DtGPDH* expression level at time 0 (before osmotic shock) was normalized as 1 for each set of experiment. Fold change (average of three independent sets of experiment) was shown based on the isotonic control group (2 M to 2 M) at the same time points. *DtTUB* was used as the endogenous reference.

5.3.5 Generation and selection of the *DtMAPK* knock-down strains

The *DtMAPK*-RNAi plasmid was constructed as described in section 5.2.5, and the construct was transformed into *D. tertiolecta* using the *Agrobacterium* mediated transformation method. About 96 colonies (1 96-well plate) were picked and cultured for DNA extraction, and then selected by genotyping PCR. A 397-bp gene fragment could be amplified as PCR products in positive colonies, as shown in Figure 5-5. Genotyping-positive transformants showing thick band of PCR

products (similar as Lane 6 or Lane 8 in Figure 5-5) were selected (S1-S14) and subjected to further selection by QRT-PCR (Figure 5-6).

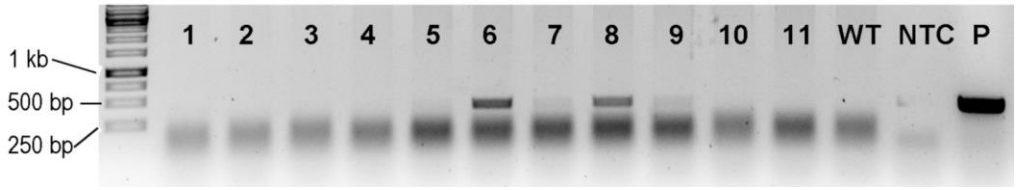

Figure 5-5 Genotyping PCR of *DtMAPK*-RNAi transformed colonies
Lane 1-11: colonies; WT: *D. tertiolecta* wild-type; NTC: non-template control; P: positive control.

The selected transformants (S1-S14), together with wild-type, were then treated with hyper-osmotic shock. The relative expression levels of *DtMAPK* of all these strains were determined at 2 hours after osmotic shock as the expression level of *DtMAPK* in *D. tertiolecta* wild-type could be highly up-regulated 2 hours after the hyper-osmotic shock from 2 M to 4 M NaCl media (Figure 5-3 A). Figure 5-6 presents the relative expression level of *DtMAPK* in different transformed strains as well as wild-type. As can be seen, some of the transformants would have similar osmo-responses to wild-type, i.e. expression of *DtMAPK* could be up-regulated upon 2 M to 4 M hyper-osmotic shock, while some other transformants, especially S9, displayed no such obvious up-regulation on the gene expression level. Besides, when comparing the 2 M isotonic controls of all these strains, *DtMAPK* expression level in S9 was also lower than that of wild-type. This suggests that S9 may be a successful *DtMAPK* gene knock-down strain. The strain S9 was then named MR2 and subjected to further investigations on its osmo-regulation of gene expression as well as the glycerol production.

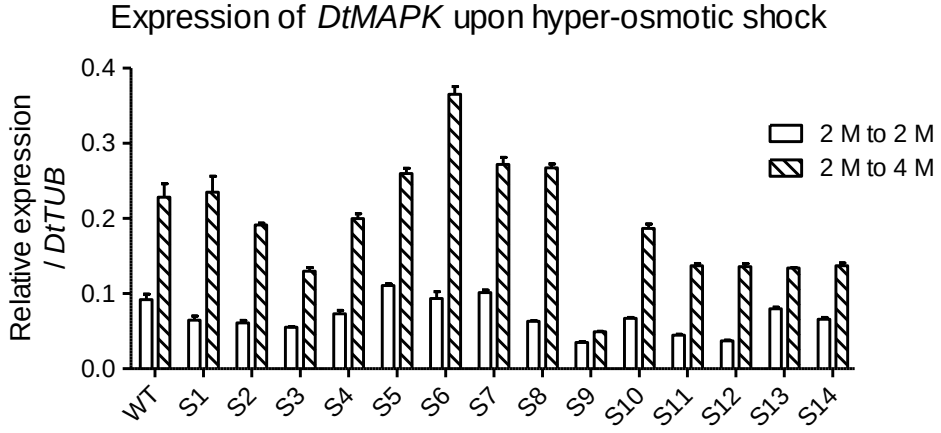

Figure 5-6 QRT-PCR results for *DtMAPK* knock-down transformants selection
WT: *D. tertiolecta* wild-type; S1-S14: 14 genotyping-possitive transformants selected by genotyping PCR. 2 M to 2 M (unshaded), isotonic control; 2 M to 4 M (shade), cells were shifted from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl for treatment of hyper-osmotic shock. Samples were taken at 2 hours after treatment. *DtTUB* was used as the endogenous reference. Values shown are means of technical triplicates.

5.3.6 Expression of *DtMAPK* in the *DtMAPK* knock-down strain MR2 upon osmotic shock

The knock-down effect of *DtMAPK* gene was confirmed in MR2 by triplicate hyper-osmotic shock experiments conducted in parallel. The relative expression of *DtMAPK* in both *D. tertiolecta* wild-type and MR2 after hyper-osmotic shock from 2 M to 4 M NaCl was shown in Figure 5-7. It is already known that *DtMAPK* expression at 2 hours upon hyper-osmotic shock could be up-regulated in *D. tertiolecta* wild-type and then slowly returned to baseline level after 4 hours (Figure 5-3 A). Here Figure 5-7 also shows a consistent result that *DtMAPK* was 4 times up-regulated in wild-type 2 hours after hyper-osmotic shock, and less than three times up-regulated 4 hours after hyper-osmotic shock. At the same time, MR2 has no significant difference on *DtMAPK*

expression level after hyper-osmotic shock treatment. Determination of the gene expression of MR2 upon osmotic shock displayed similar *DtMAPK* mRNA level to the isotonic control after 2 hours or 4 hours hyper-osmotic stress exposure. Moreover, when comparing the isotonic control groups (2 M to 2 M) where wild-type or MR2 cells were not treated with osmotic shock, Figure 5-7 also shows that *DtMAPK* expression in MR2 was always slightly lower than that in *D. tertiolecta* wild-type. These findings demonstrate that *DtMAPK* gene expression has been knocked down successfully in MR2.

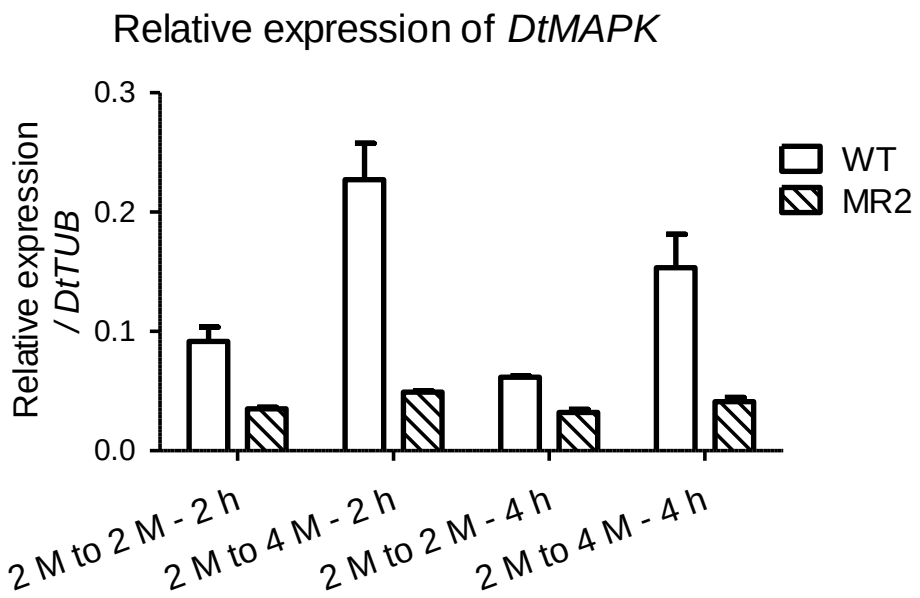

Figure 5-7 Expression of *DtMAPK* in the knock-down strain MR2 could not respond to hyper-osmotic shock
The average of three sets of experiments conducted in parallel was shown. *DtTUB* was used as the endogenous reference. WT (unshaded): *D. tertiolecta* wild-type; MR2 (shade): *DtMAPK* knock-down strain MR2. 2 M to 2 M, isotonic control; 2 M to 4 M, cells treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl. *DtMAPK* expression level was evaluated at 2 hours (A) and 4 hours (B) after treatment.

5.3.7 Expression of *DtGPDH* in the *DtMAPK* knock-down strain MR2 upon osmotic shock

With the attempt to evaluate whether *DtGPDH* could be regulated by expression of *DtMAPK*, *DtGPDH* expression level was determined in the *DtMAPK* knock-down strain MR2. Figure 5-8 shows the relative expression level of *DtGPDH* gene of MR2 2 hours and 4 hours after hyper-osmotic shock from 2 M NaCl to 4 M NaCl. As can be seen, expression of *DtGPDH* was inhibited in the *DtMAPK* gene knock-down strain MR2 compared to wild-type. MR2 had lower *DtGPDH* expression level than the wild-type in both the isotonic control group and the 2 M to 4 M hyper-osmotic shock treatment group. Since the inhibition of *DtGPDH* expression could be caused by the gene knock-down effect of *DtMAPK*, this suggests that expression of *DtGPDH* in *D. tertiolecta* could be regulated by *DtMAPK* gene, thus a possible osmo-regulatory mechanism for glycerol synthesis.

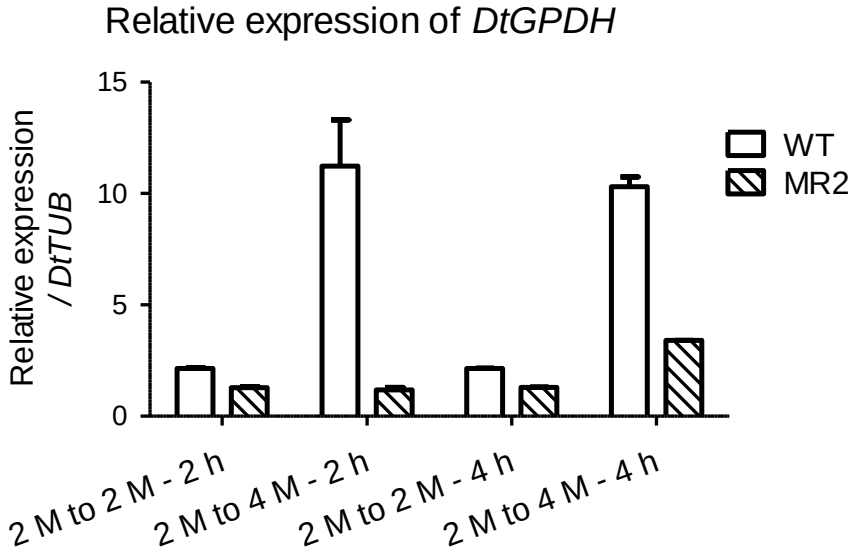

Figure 5-8 Relative expression of *DtGPDH* gene in response to hyper-osmotic shock in the *DtMAPK* knock-down strain MR2
The average of three sets of experiments conducted in parallel was shown. *DtTUB* was used as the endogenous reference. WT (unshaded): *D. tertiolecta* wild-type; MR2 (shade): *DtMAPK* knock-down strain MR2. 2 M to 2 M, isotonic control; 2 M to 4 M, cells treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl. *DtGPDH* expression level was evaluated at 2 hours (A) and 4 hours (B) after treatment.

5.3.8 Osmo-regulation of glycerol production by the *DtMAPK* knock-down strain MR2

In order to demonstrate the regulation effect of *DtMAPK* on the glycerol production, glycerol content upon hyper-osmotic shock was evaluated. Because that the cells were transferred to fresh media for experimental treatments, the extracellular glycerol concentration in the media was negligible compared to the intracellular glycerol concentration initially upon the transfer. Therefore, only intracellular glycerol concentration was shown here. Figure 5-9 describes the intracellular glycerol content measured in MR2 as well as *D. tertiolecta* wild-type 2 hours and 4 hours after hyper-osmotic shock treatment from 2 M NaCl to 4 M NaCl. The data suggests that MR2 had a slower response on its glycerol content to external osmotic pressure compared to wild-type.

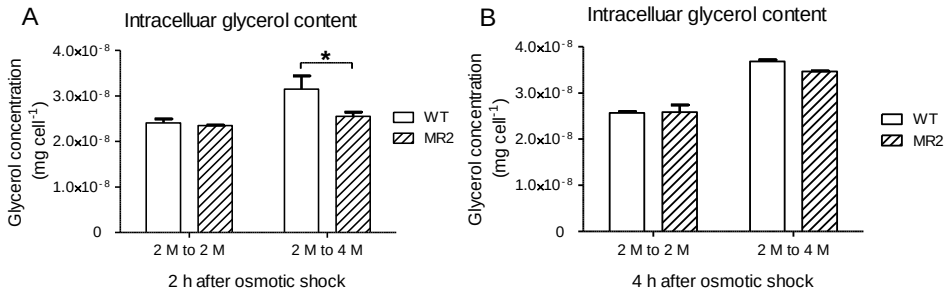

Figure 5-9 Intracellular glycerol content of the *D. tertiolecta* wild-type and MR2 in response to osmotic shock

A, glycerol content measured 2 hours after treatment; B, glycerol content measured 4 hours after treatment. WT (unshaded): *D. tertiolecta* wild-type; MR2 (shade): *DtMAPK* knock-down strain MR2. 2 M to 2 M: isotonic control; 2 M to 4 M: cells treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl. The average of three sets of experiments conducted in parallel was shown.

It is shown in Figure 5-9 that the intracellular glycerol content of *D. tertiolecta* wild-type treated with 2 M to 4 M hyper-osmotic shock was generally higher than the 2 M isotonic control. However, MR2 had significantly lower glycerol content than wild-type 2 hours after hyper-osmotic shock treatment from 2 M NaCl to 4 M NaCl, while the glycerol concentration was the same with MR2 cells in 2 M isotonic control group (Figure 5-9 A). The results suggest that the *DtMAPK* knock-down strain MR2 had different short-term (within 2 h) response to hyper-osmotic shock on its glycerol production compared to *D. tertiolecta* wild-type. However, the intracellular glycerol content determined 4 hours after treatment displayed no obvious difference between wild-type and MR2, no matter under hyper-osmotic shock or isotonic conditions (Figure 5-9 B). This could be explained from Figure 5-8 B that the *DtGPDH* gene was still slightly up-regulated in the hyper-osmotic shock treated MR2 cells 4 hours after treatment when comparing to MR2 cells treated with 2 M to 2 M isotonic condition, even though the expression level of *DtGPDH* in MR2 cells was still inhibited compared to wild-type. The slightly increase of *DtGPDH* expression could trigger the glycerol synthesis in MR2 cells undergone hyper-osmotic stress, which leads to the increase of glycerol production as a normal response to hyper-osmotic stress (Figure 5-9 B).

The regulation of glycerol synthesis in *D. tertiolecta* upon osmotic shock could be a complicated biological process involving many factors. To exclude the possibility that the difference between MR2 and wild-type on osmo-regulation of glycerol production was caused by the difference on cell growth rate, the cell numbers were counted during the osmotic shock treatment process. Fold change of cell number after 24 hours growing in the fresh media was calculated. As shown in Figure 5-10, cell growth of MR2 has no significant difference with *D. tertiolecta* wild-type in response to osmotic shock. Thus the different osmo-responses of glycerol production were not resulted from the difference on cell growth. Therefore, it could be confirmed that the glycerol production by MR2 has a different response to hyper-osmotic shock from that of *D. tertiolecta* wild-type. These findings strengthen the proposal that the glycerol production could be regulated by a Hog-like MAPK cascade signalling pathway.

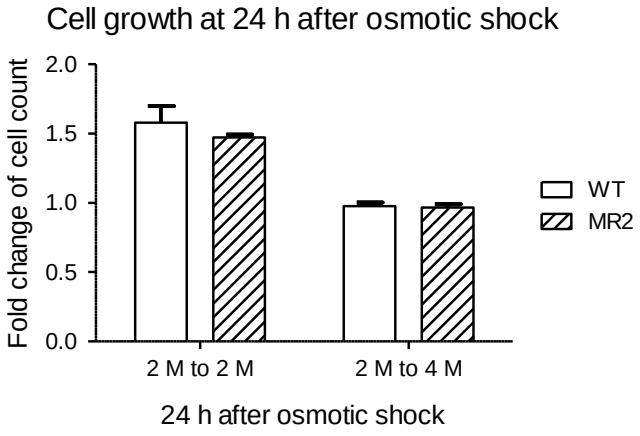

Figure 5-10 Cell growth of *D. tertiolecta* wild-type and MR2 after 24 hours exposure of osmotic stress
The average fold change of cell numbers counted in three sets of experiments was shown. WT (unshaded): *D. tertiolecta* wild-type; MR2 (shade): *DtMAPK* knock-down strain MR2. 2 M to 2 M: isotonic control; 2 M to 4 M: cells

treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl.

5.3.9 Cell size variation of the *DtMAPK* knock-down strain MR2 in response to hyper-osmotic shock

To compare the osmo-response to osmotic shock of the *DtMAPK* gene knock-down strain MR2 with *D. tertiolecta* wild-type, cell size changes under osmotic shock conditions were measured in both strains. Figure 5-11 shows the cell biovolume of MR2 and wild-type 24 hours after osmotic shock treatment. As can be seen, MR2 had a similar cell biovolume to wild-type when subjected to isotonic treatment of 2 M NaCl medium.

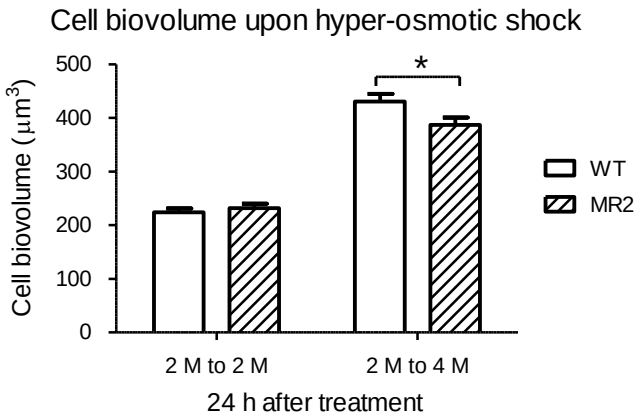

Figure 5-11 Cell biovolume of the *DtMAPK* gene knock-down strain MR2 upon hyper-osmotic shock
WT (unshaded): *D. tertiolecta* wild-type; MR2 (shade): *DtMAPK* knock-down strain MR2. 2 M to 2 M: isotonic control; 2 M to 4 M: cells treated with hyper-osmotic shock from ATCC-1174 DA medium containing 2 M NaCl to medium containing 4 M NaCl. Cell biovolume were measured 24 hours after osmotic shock. Values shown for each treatment are means of 30-40 cells. Independent sample t test were used for statistical analysis ($n >= 33$, $p = 0.03$).

As described in the previous study of Chapter 3, the cell size of *D. tertiolecta* wild-type could recover within 24 hours after the cells were

shifted from 2 M NaCl to 4 M NaCl osmotic condition. The intracellular glycerol concentration could have reached the balanced level to that of the external osmolarity by then. In this study, cell biovolume of MR2 was significantly ($p = 0.03$) smaller than wild-type after 24 hours exposure to hyper-osmotic stress from 2 M NaCl to 4 M NaCl. This observation suggests that the MR2 cells could have slow recovery process after osmotic shock treatment, or that partial of MR2 cells could not fully recovered after hyper-tonic shock treatment. Since the *DtMAPK* gene was knocked down in MR2, this finding indicates that the expression of *DtMAPK* gene was essential for the fast recovery of cell size in response to osmotic shock, possibly due to slower rate of glycerol synthesis in the MR2 as shown in Figure 5-9 A.

5.4 Discussion

5.4.1 The isolated *DtMAPK* may be different from previously reported MAPKs in *Dunaliella*

Dunaliella adapt to hyper-osmotic environments by increasing the intracellular glycerol content. Previous work has shown that MAPK-like proteins could mediate the responses to hyper-tonic shock in *D. viridis* (Jimenez, Berl et al. 2004), and antibodies specific to yeast MAPK Hog1 and mammalian MAPK p38 could recognize proteins and protein motifs that were phosphorylated and up-regulated in *D. viridis* when exposed to hyper-osmotic stress. Also, treatment with human p38 inhibitor could markedly impair the adaptation of *D. viridis* to osmotic

stress. However, in this study, the *DtMAPK* of *D. tertiolecta* could not be recognized by the antibodies reported in the study of *D. viridis* (Jimenez, Berl et al. 2004). This suggests that *DtMAPK* could be a MAPK encoding gene different from that of *D. viridis* and yeast. Moreover, treatment on *D. tertiolecta* wild-type cells of the same MAPK-inhibitor specific to human p38 had no effect on the glycerol production as well as the adaptation process after osmotic shock. These findings further suggest that *D. tertiolecta* might have different MAPK proteins from *D. viridis* and yeast.

On the other hand, a MAPK gene (*DsMPK*) has been reported in *D. salina* (Lei, Qiao et al. 2008). Alignment with *DsMPK* and *DtMAPK* displayed a 98% similarly on their amino acid sequences. This suggests that *DtMAPK* is highly conserved and has a close relationship with *DsMPK*. However, studies on *DsMPK* showed that the gene was down regulated upon hyper-osmotic shock (Lei, Qiao et al. 2008) while *DtMAPK* in *D. tertiolecta* was found to be up-regulated upon hyper-osmotic shock and suppressed upon hypo-osmotic shock (Figure 5-3). To further confirm that, a hypo-osmotic shock treatment experiment were conducted using *D. salina*, and the result suggested that *DsMPK* was slightly up-regulated by hypo-osmotic shock (data not shown), which supported the result of the *D. salina* report (Lei, Qiao et al. 2008). These findings indicate that *D. tertiolecta* could have different regulatory mechanisms in response to osmotic shock treatments from that of *D. salina*.

Overall, the above information indicates that *DtMAPK* had different behavior in *D. tertiolecta* in response to osmotic stress. It could be suggested that *D. tertiolecta* may possess different MAPK proteins and have a different regulating mechanisms in response to osmotic pressure.

5.4.2 Expression of *DtGPDH* could be regulated by *DtMAPK*

As the GPDH homologue has been identified in *D. tertiolecta*, its gene expression profile was determined under various osmotic stress conditions together with the identified *DtMAPK* gene. The results showed that the expression level of both *DtGPDH* and *DtMAPK* in *D. tertiolecta* cells were positively correlated to the external salinities (Figure 5-2). Since the glycerol production was also positively correlated to the external salinity (Chapter 3), it could be proposed that the glycerol production is controlled by *DtGPDH* gene and the expression level of *DtGPDH* might be regulated by *DtMAPK*.

Further studies on the expression of *DtGPDH* and *DtMAPK* in response to hyper-osmotic shock demonstrated that both genes were transiently up-regulated upon hyper-osmotic shock treatment. However, the up-regulation of *DtMAPK* occurred 1-4 hours after osmotic shock (Figure 5-3 A), while the expression of *DtGPDH* started to increase since 2 h after hyper-osmotic shock and the highest induction of *DtGPDH* happened 4-8 hours (Figure 5-4). It is suggested that *DtGPDH* gene expression is downstream that of *DtMAPK* upon osmotic shock, and there is a delay in its up-regulation.

5.4.3 *DtMAPK* was involved in mediating the short-term osmo-response of *D. tertiolecta*

GPDH enzyme has been considered to be the key enzyme for glycerol synthesis and its encoding genes have been isolated in *D. salina* (He, Qiao et al. 2007) and *D. viridis* (He, Meng et al. 2009). The expression level of the isolated *DtGPDH* gene in *D. tertiolecta* was found to be positively correlated to the external salinities (Figure 5-2 A). Besides, the glycerol production was proportional to the external salinity as discussed in Chapter 3. Therefore, it can be suggested that *DtGPDH* is also responsible for the glycerol synthesis in *D. tertiolecta* for the osmotic adaptation.

Furthermore, *DtMAPK* gene knock-down strain MR2 displayed a different osmo-regulation pattern of glycerol production upon hyper-osmotic shock (Figure 5-9). Lower production of glycerol was observed in MR2 cells treated with hyper-osmotic shock, while no difference was observed in the cells with isotonic treatment. These results may suggest that *DtMAPK* was essential for the fast initial regulation of the glycerol synthesis in response to osmotic shock. Therefore, it could be confirmed that *DtMAPK* was involved in the osmo-regulation of glycerol synthesis in *D. tertiolecta* by regulating the expression of *DtGPDH* gene.

MAPK-like proteins have been reported to mediate the response of *D. viridis* to hyper-tonic shock (Jimenez, Berl et al. 2004). It could be suggested that a yeast HOG-like MAPK cascade could be involved in

osmo-regulation in *D. tertiolecta* upon osmotic stress. The MAPK encoding gene *DtMAPK* isolated sheds light on this presumptive MAPK cascade responsible for the osmo-regulation in *D. tertiolecta*. Glycerol production upon osmotic shock could be affected by *DtMAPK* gene (Figure 5-9), and the expression of *DtGPDH* gene, which encodes the key enzyme for glycerol synthesis, could be regulated by *DtMAPK* (Figure 5-8). These findings suggested that *DtMAPK* should be involved in osmo-regulation on glycerol synthesis of *D. tertiolecta*. In addition, the cell size measured in the *DtMAPK* gene knock-down strain MR2 could not fully recovered after hyper-osmotic shock treatment (Figure 5-11). This could be caused by the slower intracellular glycerol accumulation rate, suggesting that *DtMAPK* was essential for the activation of glycerol synthesis in *D. tertiolecta* in response to osmotic shock.

Overall, the findings strongly suggested that *DtMAPK* was essential for short-term osmo-regulation in *D. tertiolecta*. There is a high chance that a HOG-like signalling pathway could be involved regulating the osmo-response of *D. tertiolecta*.

5.4.4 Other possible regulating mechanisms in *D. tertiolecta*

It is possible that *D. tertiolecta* has more than one pathway to control the glycerol production and osmoregulation. Other mechanisms or core molecules have been considered to function collaboratively or take part in the osmo-regulation in *Dunaliella*.

Investigation (Chen, Chen et al. 2011) verified that the calcium channels and intracellular Ca^{2+} concentration in *D. salina* could respond rapidly to extracellular osmotic change; Changes of glycerol production as well as GPDH activity depended on the influx of Ca^{2+} via Ca^{2+} channels. In *D. tertiolecta*, a Ca^{2+}-dependent protein kinase (CDPK) has been purified, which could be activated by Ca^{2+} under osmotic stress (Yuasa and Muto 1992). Hence a Ca^{2+}-mediated osmotic signalling pathway was suggested in *D. salina* in response to osmotic stress.

5.5 Conclusion

Sequence of the isolated MAPK encoding gene *DtMAPK* bears high similarities to other MAPKs in green algae. As the expression of *DtMAPK* responded to the changes of external osmotic pressure, it can be suggested that the isolated *DtMAPK* may be involved in the osmo-regulatory mechanism of *D. tertiolecta*.

Knock-down of *DtMAPK* gene resulted in the inhibition of *DtGPDH* in *D. tertiolecta* upon osmotic shock. This indicated that expression of *DtGPDH* could be regulated by *DtMAPK*. As *DtGPDH* is considered encoding the key enzyme GPDH for glycerol synthesis in *D. tertiolecta*, and the glycerol production by the *DtMAPK* knock-down cells had a slower response to hyper-osmotic shock, it can be concluded that glycerol production could be regulated in *D. tertiolecta* by *DtMAPK* via the regulation on the expression of *DtGPDH* gene. Moreover, the *DtMAPK* knock-down strain MR2 cells could not fully recover after

hyper-osmotic shock treatment, which suggested that *DtMAPK* might be essential for the osmotic adaptation in *D. tertiolecta*. Both the glycerol synthesis and the cell size variation in response to osmotic shock could be affected by knock-down of *DtMAPK* gene. Therefore, the isolated *DtMAPK* is essential in the osmo-regulatory mechanism of *D. tertiolecta*. The study also demonstrated that expression of *DtGPDH* could be regulated by *DtMAPK* for the first time, suggesting a yeast HOG-like signalling pathway is involved in *D. tertiolecta* mediating its osmo-regulation under osmotic stress conditions.

CHAPTER 6 CONCLUSION AND FUTURE PROSPECTS

Aims of this project were to study the osmo-regulatory mechanisms of *D. tertiolecta* in response to osmotic shock treatments. The study included not only the physiological investigations on the osmo-responses of *D. tertiolecta* upon various osmotic pressures, but also the molecular evidences for the osmo-regulatory mechanisms.

6.1 *D. tertiolecta* is an Effective CO_2 Scrubber

Investigations on the physiological responses of *D. tertiolecta* cells demonstrated that *D. tertiolecta* could respond to osmotic shock treatments by varying cellular metabolic activities as well as the cell structure rapidly. This detailed physiological study provided new information on the cellular behavior of *D. tertiolecta* in the osmo-regulation process.

D. tertiolecta glycerol production was found to be up-regulated by hyper-osmotic stress and was proportional to the external salinity. At the same time, the large amount of extracellular glycerol produced under high osmolarity can be an effective carbon sink for the photosynthetic CO_2 assimilation. The extracellular glycerol production was not physically limited by cell size, and it could also lower the cost of glycerol harvesting process as cell disruption is not needed. These findings indicate the potential of using *D. tertiolecta* for commercial

production of glycerol as well as the carbon pool for the atmospheric CO_2 capture.

6.2 Pathways Involved in the Osmo-regulation in *D. tertiolecta*

The molecular basis of osmo-regulatory mechanisms in *D. tertiolecta* has not been well studied. With the purpose to identify the pathways regulating the osmotic responses of *D. tertiolecta*, two key enzymes PFK and MAPK involved in the regulation of glycerol synthesis were characterized.

Since the whole genome sequence of *D. tertiolecta* is not available, *MAPK* and *PFK* homologue in *D. tertiolecta* were cloned by employing the RACE technology, and the isolated cDNA sequences were named *DtMAPK* and *DtPFK*. Gene sequence analysis and protein alignment results show that both DtMAPK and DtPFK have close relationship with their homologous proteins in other microalgae. This finding suggests that these genes in algae are highly conserved, and *D. tertiolecta* could be a good model for the study of salt tolerance mechanisms and stress responses.

As PFK has been reported to be an important checkpoint enzyme for the activation of glycerol synthesis in *D. salina* (Chitlaru and Pick 1991), PFK in *D. tertiolecta* was studied on its gene expression. However, the results suggested that deficiency of *DtPFK* gene in *D. tertiolecta* had no obvious effects on the glycerol production in response to osmotic

pressure. The results were different from the study of the budding yeast (Dihazi, Kessler et al. 2004), where the yeast cells lacking PFK were not able to grow under hyper-tonic stress. It could be suggested that *DtPFK* may not be the bottle-neck involved in the osmo-regulation of glycerol synthesis in *D. tertiolecta*. Considering that the PFK catalyzing the rate-limiting step of glycolysis pathway for the break-down of glucose from starch degradation, as well as that the determined starch content in *D. tertiolecta* cells were extremely low, it could be concluded that starch break-down may not contribute to the glycerol synthesis of *D. tertiolecta* in response to osmotic stresses as the carbon provider under the experimental conditions.

In addition, a MAPK-like protein mediated signalling pathway was demonstrated in this project that could be involved in regulating the glycerol synthesis under osmotic stress. The study found that expression of *DtGPDH*, which encodes the key enzyme for glycerol synthesis, could not be activated by hyper-osmotic shock in the *DtMAPK* gene knock-down mutant, which led to the inhibition of glycerol synthesis in a short period of time after treatment. These findings provide direct evidence that *DtGPDH* could be regulated by *DtMAPK*, and *DtMAPK* could be involved in osmo-regulation in *D. tertiolecta* under osmotic shock. The presumptive osmo-regulatory mechanism that the glycerol synthesis was activated by *DtGPDH* while expression of *DtGPDH* was regulated by *DtMAPK* was demonstrated for the first time in *D. tertiolecta*.

The study shed light on the osmo-regulatory mechanism of *D. tertiolecta* glycerol synthesis under osmotic pressures. The findings suggested that *DtMAPK* was transiently up-regulated after hyper-osmotic shock, and the osmo-regulation on glycerol production in the *DtMAPK* knock-down cells was only inhibited for a short period of time after osmotic shock. A possible explanation could be that *DtMAPK* was responsible for short-term osmo-regulation in *D. tertiolecta*, and other mechanisms in *DtMAPK* knock-down cells could be also involved in regulating its osmo-responses.

6.3 Suggestions for Future Work

The two core enzymes PFK and MAPK of the regulatory pathways for glycerol synthesis were studied. *DtMAPK* appeared to be involved in the osmo-regulation in *D. tertiolecta* upon osmotic stress. To fully understand the function of DtMAPK protein as a regulator for glycerol synthesis in *D. tertiolecta*, study on the translational level of DtMAPK is necessary. Currently commercial antibodies specific to the MAPK homologues, p38 and hog1 were found not working for *D. tertiolecta*. Therefore, DtMAPK specific antibodies need to be generated for its functional study.

DtMAPK has been demonstrated to mediate the osmo-regulation in *D. tertiolecta* and trigger its glycerol synthesis. As a complicated biological process, other molecules could function collaboratively in this signalling pathway. This project could be extended to identify other components in this signal transduction process. For example, it is reasonable to

assume that sensors for the osmo-regulatory mechanism reside in the plasma membrane. Some cell surface proteins have been reported to response to high salinity and play potential roles in the osmo-regulation in *D. salina* (Sadka, Himmelhoch et al. 1991; Fisher, Pick et al. 1994). Plasma membrane proteome study has also been done in *D. salina* (Katz, Waridel et al. 2007). For more details of the *DtMAPK*-mediated signalling pathway in *D. tertiolecta*, such work could be conducted, and the osmo-sensors could be identified among the salt-induced membrane located proteins in *D. tertiolecta*.

On the other hand, this project has demonstrated that *D. tertiolecta* could be an effective atmospheric CO_2 scrubber and the abundant extracellular glycerol synthesis could represent an efficient carbon sink for photosynthetic CO_2 assimilation. Gene modification approach such as over-expression of *DtMAPK* could be employed in the future to generate transgenic *Dunaliella* strains with enhanced CO_2 fixation ability as well as high glycerol productivity.

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